Neuroendocrine responses to stress in a teleost, Oncorhynchus mykiss

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Award date:
2000

Awarding institution:
University of Bath

NEUROENDOCRINE RESPONSES TO STRESS IN A TELEOST, *ONCORHYNCHUS MYKISS*

Submitted by Ben Gilchriest
for the degree of PhD
of the University of Bath
2000

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ACKNOWLEDGEMENTS

Thanks go to all those who aided in any, way, shape or form towards the completion of this thesis. In particular to my supervisor Dr.Bridget Baker for advice, constructive criticism, and the odd push when required, my family for support both financial and otherwise, and Dr.David Tipping for sharp and concise comments that were a source of continuous assistance. Also thanks to Chris Davey, Jim Knight and Ray Dickson for their unerring assistance with the aquariums and BBSRC for financing this work.

I would also like to stretch a debt of gratitude to M; never contributing directly but essential nonetheless. A final thank you goes to Justin, Jonny, Ruairi, and Graham for even more obscure and indirect support.

Summary

This study on the rainbow trout (*Oncorhynchus mykiss*) employed quantitative *in situ* hybridisation to investigate the response to acute and chronic stress of arginine vasotocin (AVT) and isotocin (IT) transcripts in the hypothalamic parvo- and magno- cellular neurones, and pituitary pro-opiomelanocortin (POMC) mRNAs in the melanotropes and corticotropes. Some experiments also examined the influence of cortisol negative feedback on these cells. The possibility that the melanin-concentrating hormone (MCH) might influence gene transcripts was also investigated.

A quantitative *in situ* hybridisation method for measuring AVT and IT mRNA values was first established. The changes of transcripts over a 24 hour cycle were then monitored in unstressed trout for the purposes of planning the timing of stress experiments. Parvocellular AVT mRNA plateaued between 10.00h and 22.00h, during which period all future stressors were applied. Pituitary POMC transcripts in the melanotropes followed a marked diurnal pattern, however, the two genes, POMC-A and POMC-B, did not change in synchrony, indicating independent control.

Some acute stressors enhanced parvo-AVT transcript abundance, confirming the involvement of these neurones in the stress response. However, this response was not seen consistently; furthermore, daily repetition of even mild stresses depressed parvo-AVT mRNA. Possible reasons for this are discussed. POMC transcripts in both pituitary cell types varied in their response to both acute and chronic stress. Parvo-IT and magno-AVT transcripts failed to show any response to acute stress.

When cortisol-coated pellets were fed to trout, corticotrope POMC mRNA was more sensitive to the exogenous steroid than AVT transcripts which required a five-fold higher dosage in order to suppress below basal levels.

Contrary to expectations, AVT transcripts were lower in black- than white- reared trout; possible explanations for this are discussed, however, these results question the value of rearing fish long term on a white background in order to restrain the stress response.

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1. Gilchriest, B. J., Tipping, D. R., Levy, A., and Baker, B. I. (1998). Diurnal changes in the expression of genes encoding for arginine vasotocin and pituitary pro-opiomelanocortin in the rainbow trout (*Oncorhynchus mykiss*): correlation with changes in plasma hormones. *J. Neuroendocrinol.* **10:** 937-943.

2. Gilchriest, B. J., Tipping, D. R., Levy, A., and Baker, B. I. (1999). 'The influence of acute or chronic stress of different durations on MCH mRNA abundance in rainbow trout (*Oncorhynchus mykiss*)'. In: *Recent Developments in Comparative Endocrinology and Neurobiology*, pp 135-139. Ed(s). Roubos, Wendelaar-Bonga, Vaudry & Loof. Nijemgen.

3. Gilchriest, B. J., Tipping, D. R., Hake, L., Levy, A., and Baker, B. I. (2000). The effects of acute and chronic stresses on vasotocin gene transcripts in the brain of the rainbow trout (*Oncorhynchus mykiss*). *J. Neuroendocrinol.* **12:** 795-801.

Chapter 1

General Introduction

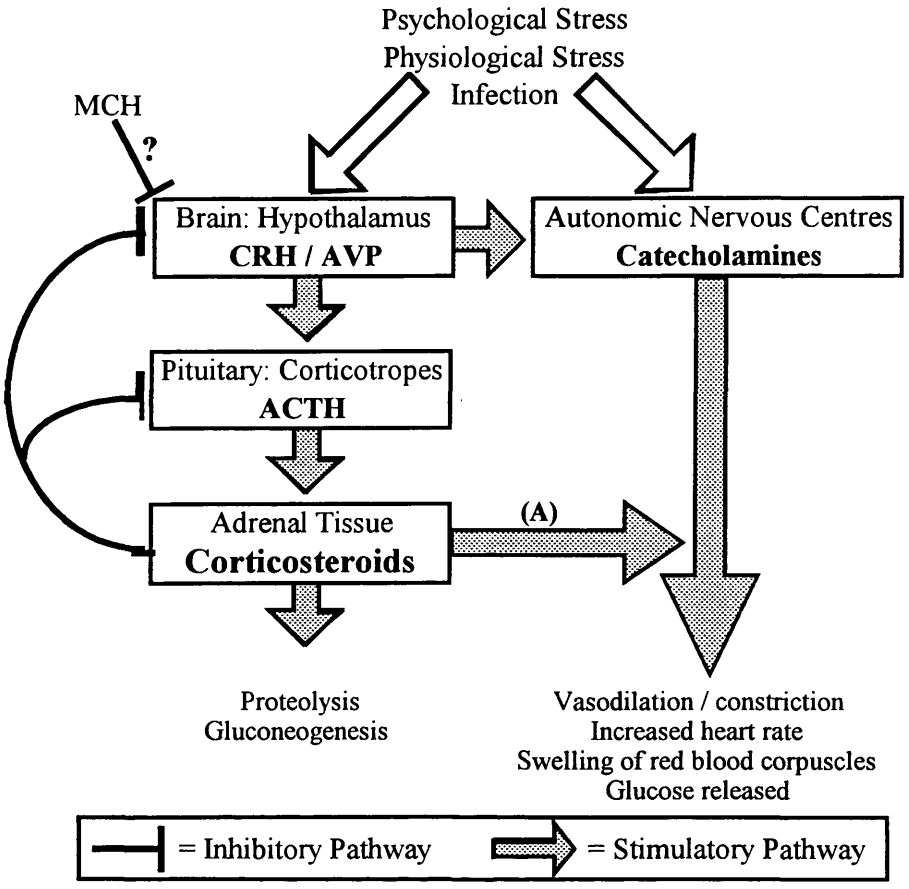
1.1 Overview

Stress is a general term that describes a real or perceived threat to the homeostatic state (Pickering, 1981). Survival is dependant upon this ability to respond to an immense variety of challenges. In vertebrates the stress response is characterised by the release of catecholamines from the sympathetic nervous system within a few seconds followed, several minutes later, by corticosteroid release from adrenal tissue. These hormones act on cardiovascular and respiratory systems to increase heart rate, blood pressure, and respiration rate, as well as increasing plasma oxygen and glucose and so essentially preparing the animal for a fight or flight reaction.

Generally the severity and duration of the stress is reflected in the magnitude of the corticosteroid response (Barton & Iwama, 1991), this being one of the end point hormones in the stress cascade (see Figure 1.1). In fish the plasma concentration of the main corticosteroid, cortisol, has been commonly used as a monitor of stress. However, changes in plasma cortisol concentration do not necessarily correspond to changes in release as the circulating level is also affected by clearance or turnover rate. In addition, with repeated stress cortisol habituation can occur with plasma corticosteroid levels returning to basal levels despite the stress, and activation of the hypothalamus, continuing. Cortisol release can also be stimulated by other factors such as urotensin or angiotensin II, independently of the hypothalamic response to stress. Furthermore, since plasma cortisol can rise within a few minutes, the concentration at the time of sampling could be indicative of a more recent disturbance than the result of the tested stress paradigm *per se*. Under certain stressful conditions, such as confinement when water quality is poor, plasma cortisol release can also be inhibited (Pickering & Pottinger, 1987*a*).

In recent years researchers have followed adrenocorticotropic hormone (ACTH) plasma concentrations. This pituitary hormone stimulates cortisol release from the adrenal tissue and is therefore a closer indicator of the stress response. However, even profiles of ACTH may be misleading since it can rise rapidly (within two minutes) in response to stress (Sumpter *et al.*, 1986) which could include capture at the time of sampling.

Figure 1.1; Schematic of the cascade response to stress by the hypothalamo-pituitary-adrenal axis. (A) action on adrenergic receptors and adrenergic enzymes.



The purpose of this research was to investigate stress-induced changes of the hypothalamic neuropeptides corticotropic releasing hormone (CRH) and arginine vasotocin (AVT). These stimulate ACTH release from the trout pituitary (Baker *et al.*, 1996) and thus are central to the control of stress. In addition, it is now known that CRH has a range of effects on the sympathetic nervous system, adrenaline release and behaviour. Information about these corticotropic secretogogues in mammals is extensive (Harbuz & Lightman, 1992). In rats, CRH and arginine vasopressin (AVP), the homologue of fish AVT, respond differentially to stress depending on the duration and type of stress. However, almost nothing is known about the synthesis and secretion of these two hormones in the stress response in fish. It is for this reason they have been investigated in this context. This introductory section aims to describe the key elements

involved within this response and the extent of current knowledge and understanding of them.

1.2 The Hypothalamus

The hypothalamus is the major homeostatic center of the brain. It is the basal part of the diencephalon and lies beneath the thalamus as the name implies. The floor of the hypothalamus extends downwards and makes contact with the pituitary. In mammals, peptides implicated in the control of the hypophysial hormones are transported to the pituitary via a vascular link, the hypophysial portal system (HPS) between the richly vascular median eminence, situated at the lower part of the hypothalamus, and the pituitary. In fish there is no functional hypothalamo-hypophysial portal system (Peter & Fryer, 1983) with communication between hypothalamus and pituitary achieved by direct innervation (Kaul & Vollrath, 1974).

A great deal more is known about the hypothalamo-pituitary-adrenal (HPA) axis in mammals than fish as they have been more widely studied.

1.2.1 Mammalian hypothalamic stress hormones

In the rat the main neuropeptides involved in stress are CRH, AVP, and oxytocin (OT) (Rivier & Vale, 1983). Urocortin (UC) may also be involved though its role appears to be more indirect as it mediates CRH receptor sensitivity to the CRH peptide (Bittencourt & Sawchenko, 2000). Of these, CRH and AVP are considered to be the principal regulators of the stress response (Linton *et al.*, 1985; Antoni, 1993; Bartanusz *et al.*, 1993*a* and *b*). In many mammals the former of these, CRH, is believed to be the predominant peptide involved in the stress response as it is responsible for both ACTH release (Rivier & Plotsky, 1986), and transcription of POMC (pro-opiomelanocortin), the ACTH precursor molecule (Levin *et al.*, 1989). Though AVP does weakly stimulate ACTH release (Rivier & Vale, 1983; Plotsky, 1991), it is unable to stimulate transcription of POMC, at least in rats (Levin *et al.*, 1989). AVP plays a more important role in stress through its synergistic action with CRH on release, as demonstrated *in vitro* (Gillies *et al*, 1982; Vale *et al.*, 1983) and *in vivo* (Rivier & Vale, 1983). An additional measure of control over ACTH release is achieved through changes in the expression of neuropeptide receptors on pituitary corticotropes (Hauger & Aguilera, 1992).

CRH and AVP are located in parvocellular neurones of the paraventricular nucleus (PVN) in the hypothalamus (Antoni, 1993); axons from these perikarya terminate in the median eminence where the hormones are released into the HPS. Within the PVN there are two types of CRH neurone, AVP-containing and AVP-deficient (Whitnall, 1988, 1989, 1990). Thus, approximately 50% of parvocellular CRH-positive axons and terminals in the rat also contain AVP in the normal resting state (Whitnall *et al.*, 1987). This co-localisation facilitates the synergistic action of these peptides. AVP is also located within magnocellular neurones of the PVN. These terminate in the posterior pituitary and release AVP into the circulating blood where it is involved in cardiovascular and osmotic regulation (Ishii & Urano, 1980; Nojiri *et al.*, 1986).

The efficacy and actions of CRH and AVP as described above for the rat are not universal amongst mammals. In the sheep, in direct contrast to the rat, AVP is the predominant ACTH secretogogue and can stimulate POMC transcription (Liu *et al.*, 1990; Van de Pavert *et al.*, 1997). Furthermore, AVP and CRH do not act synergistically on ACTH release, although AVP does increase corticotrope responsiveness to CRH (Madsen *et al.*, 1991).

Further to their role in the stress response, some of these hormones have certain behavioural effects. When injected intracerebroventricularly (icv) CRH has been shown to suppress feeding (Negri *et al.*, 1985; Bernier and Peter, 1999; Momose *et al.*, 1999), decrease locomotor activity and exploratory behaviour in a novel environment (Sutton *et al.*, 1982; Berridge & Dunn, 1986), cause anxiety (Dunn & File, 1987; Abreu *et al.*, 1990), inhibit masculine sexual behaviour (Sirinathsinghji, 1987), and activate the sympathetic nervous system (Garcia-Belenguer *et al.*, 1993). CRH is also believed to have a unique role in mediating stress-related behavioural responses (Koob & Heinrichs, 1999), this being of particular importance when the animal must activate pituitary and adrenal systems along with the central nervous system in response to environmental challenge. Furthermore, in rats, CRH immunoreactive receptors are widely distributed throughout the brain (Cummins *et al.*, 1983; Swanson *et al.*, 1983; DeSouza *et al.*, 1985) indicating that it may act as a neurotransmitter in addition to acting as a hypothalamic hormone (Momose *et al.*, 1999).

1.2.2 Fish hypothalamic stress hormones

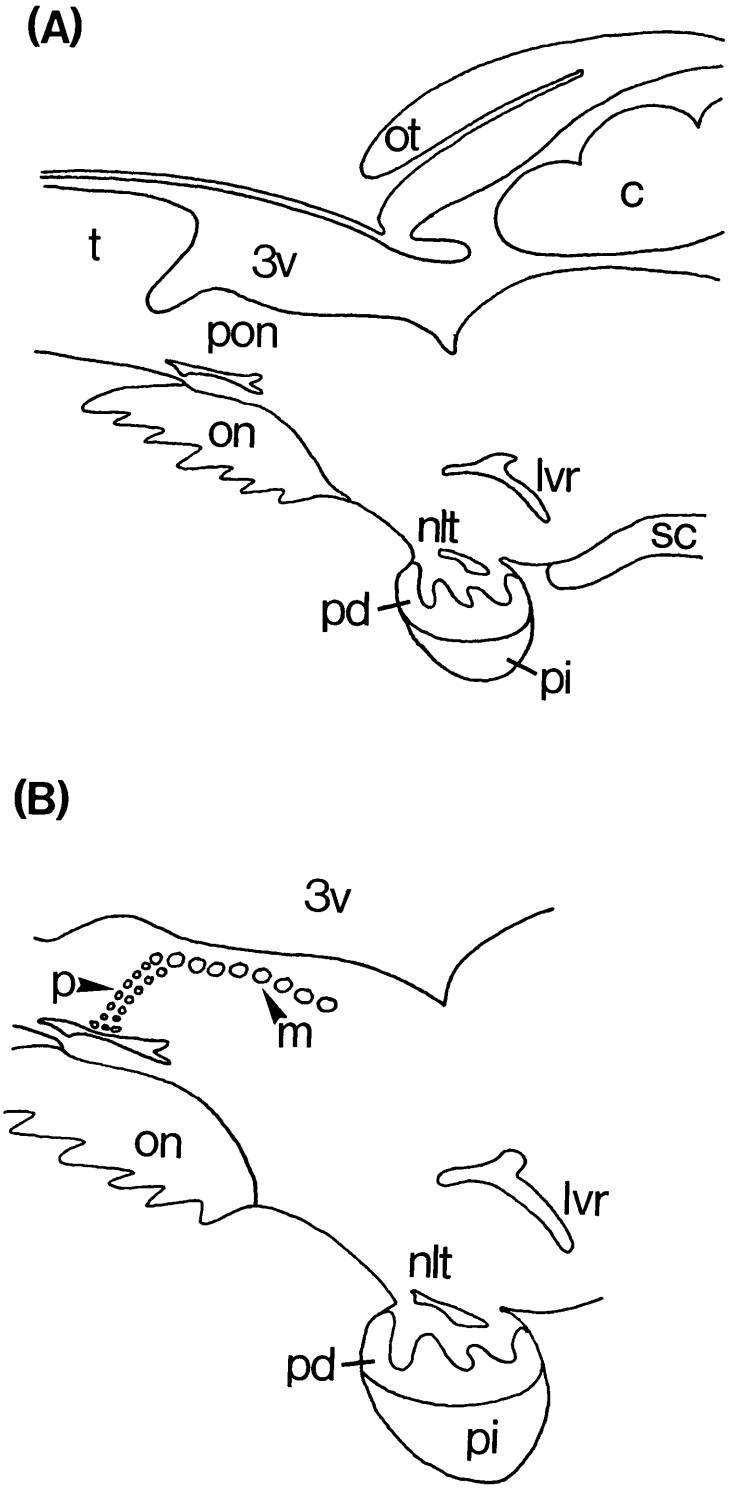
In fish there is no morphologically distinct adrenal gland, but corticosteroid producing cells are located within the head kidney interrenal tissue. Consequently the HPA is referred to as the hypothalamo-pituitary-interrenal, or HPI, axis. Although the regulation of stress at the hypothalamic level has not been widely studied in fish, homologues of AVP, CRH, OT, and UC have been identified. Of these neurohypophysial hormones amphibian mesotocin has been supposed to be derived from fish isotocin (IT) and to have been replaced in mammals by oxytocin (OT). Mammalian AVP and teleost AVT appear to have originated from a common ancestral vasotocin molecule (Hyodo *et al.*, 1991; Urano *et al.*, 1992). Mammalian urocortin appears to represent a tetrapod orthologue of urotensin-1 (U-1) in fish (Lovejoy & Balment, 1999), whilst a homologue CRH has recently been identified in a few teleost species (white sucker, *Catastomus commersoni*: Okawara *et al.*, 1988; sockeye salmon, *Oncorhynchus nerka*: Ando *et al.*, 1999; goldfish, *Carasius auratus*: Bernier *et al.*, 1999; tilapia, *Oreochromis mossabicus*: Van Enckevort *et al.*, 2000).

Of these neuropeptides IT, AVT and CRH have been shown to stimulate ACTH release from trout corticotropes though isotocin only weakly so (Pierson *et al.*, 1996). In the trout CRH and AVT produce dose dependant increases in ACTH release from intact pars distali *in vitro* with the latter less potent than the former (Baker *et al.*, 1996). Furthermore, the simultaneous addition of these two peptides has a clear synergistic effect consistent with previous studies on mammals. In contrast, in the goldfish, the combined actions of ovine CRH and AVT on the release of ACTH from dispersed pituitary cells are additive, as opposed to synergistic (Fryer *et al.*, 1985). Work on isolated goldfish pituitary cells has also shown that in addition to these neuropeptides urotensin-1 has ACTH secretogogue actions (Fryer *et al.*, 1985). However, since U-1 expression is three- to seven- fold lower than that of CRH throughout the goldfish brain (Bernier *et al.*, 1999), its importance in the control of ACTH release relative to CRH may be low. Although CRH is thought to play an important role in the control of the HPI axis in fish (Lederis *et al.*, 1994), its dominance amongst the corticotropin-releasing peptides in the neuroendocrine integration of the stress response remains to be demonstrated (Fryer, 1989; Wendelaar-Bonga, 1997).

The preoptic nucleus (PON), where most of the AVT and CRH perikarya are located in the trout, is widely accepted to be the functional homologue of the mammalian PVN. Indeed lesions of the PON suppress the corticosteroid response of goldfish to a variety of stressors (Fryer & Peter, 1975). Immunocytochemical studies have identified both parvo- and magno- cellular (see Figure 1.2) AVT and IT containing neurones with an almost identical, though independent, occurrence in the PON of the rainbow trout brain (Van den Dungen *et al.*, 1982). Work on trout, amongst other teleost species, has also found that corticotropin releasing factor (CRF)-41 peptide is located in this same region (Olivereau & Olivereau, 1988*a*, 1990*a*; Olivereau *et al.*, 1988; Matz & Hofeldt, 1999; Zupanc *et al.*, 1999). As in mammals, AVT and CRH co-localise in some of these neurones. This has been reported for a number of teleost species, including white sucker (Yulis & Lederis, 1987), goldfish (Fryer & Lederis, 1988), and eel (Olivereau *et al.*, 1988), though the relative abundance of co-localising neurones varies between species and with physiological state. A second group of immunoreactive (ir)CRH neurones has also been described in some teleost species in the nucleus lateralis tuberis (NLT) including white sucker (Yulis & Lederis, 1987), the eel (Olivereau & Olivereau, 1990*a*), sea bream (Mancera & Fernadez-Llebrez, 1995), and chinook salmon (Matz & Hofeldt, 1999).

Unlike mammals, fish lack a conventional hypophysial portal system as fibers project directly from the perikarya of the hypothalamus to terminate in the pituitary gland (Davis, 1983; Peter & Fryer, 1983) causing secretory cells of the same type to be grouped (Cambre *et al.*, 1986; Farbridge & Leatherland, 1986). The association of particular ir-fibres with particular cells thus allows one to postulate which hypothalamic neuropeptides control which pituitary hormones. The close proximity of irAVT and irCRH fibres to the melanotropes of the pars intermedia and the corticotropes of the pars distalis (Yulis & Lederis, 1987; Van den Dungen *et al.*, 1982; Olivereau & Olivereau, 1988*a*, 1990*a*; Matz & Hofeldt, 1999), suggests that they may control the activity of these two pituitary cell types. The origins of these fibres is unclear as it is not always possible to distinguish terminals from fibres of passage. Consequently it is possible that these originate in either, or both, the parvo- and magno- cellular neurones. The magnocellular AVT neurones are thought, however, to project to the pars intermedia where they release AVT into the blood and influence osmoregulation (Hyodo & Urano, 1991; Balment *et al*, 1993) and blood pressure (Le Mevel *et al.*, 1993; Warne & Balment, 1997).

Figure 1.2; Diagram showing the principal nuclei of interest and the location of parvo- and magno-cellular neurones in the rainbow trout brain (parasagittal section). (A) Overview of the location of the nuclei of interest; t = telencephalon, 3v = 3rd ventricle, ot = optic tectum, c = cerebellum, pon = pre-optic nucleus, on = optic nerve, lvr = lateral ventricular recess, nlt = nucleus lateralis tuberis, pd = pars distalis, pi = pars intermedia, sc = saccus vasculosus. (B) Magnified view showing the location of parvo- and magno-cellular neurones in the pre-optic nucleus, where p = parvocellular and m = magnocellular. The distribtuion of AVT, CRH, and IT immunoreactives overlap (see text) and covers the parvo- and magno-cellular neurones shown. Diagrams are based on information from Van den Dungen *et al.*, 1982; Olivereau *et al.*, 1988; Olivereau & Olivereau, 1988*a*, 1990*a*; Gilchriest *et al.*, 1998; Matz & Hofeldt, 1999; Zupanc *et al.*, 1999.



In situ hybridisation studies have shown that AVT and IT mRNA distribution matches that of their peptides (Hyodo & Urano, 1991) as one would expect. However, CRH mRNA displays a more patchy association with its product. In the white sucker, correlation between irCRH and CRH mRNA location is poor (Okawara *et al.*, 1992), though some neurones may be peptide storing and thus biosynthetically inactive, and vice-versa.

1.3 Factors Regulating CRH and AVT

1.3.1 The effects of stress

In rats the response of CRH and AVP to stress has been more widely studied, and thus a greater comprehension of their roles has been gained than is available in fish. CRH is generally accepted as the primary neuropeptide responsible for acute (i.e. single) stress-induced ACTH release from the anterior pituitary (Harbuz & Lightman, 1989*a*; Lightman & Harbuz, 1993; Makino *et al.*, 1995). Many acute stresses, both psychological or physiological, increase CRH mRNA abundance (see Table 1.1). Although increases in AVP mRNA in response to acute stress are less marked they do nonetheless occur (see Table 1.2).

Table 1.1; Examples of acute stressors that induce upregulation of CRH mRNA in the rat hypothalamus.

Stress Type	Stress Category	Reference (s)
Immobilisation	Psychological	Imaki *et al.*, 1992
Restraint	Psychological	Harbuz *et al.*, 1994; Kalin *et al.*, 1994
Swimming	Psychological	Harbuz & Lightman, 1989*b*
Footshock	Psychological	Lightman & Young, 1988
Hypotensive haemorrhage	Physiological	Darlington *et al.*, 1992
Insulin-induced hypoglycaemia	Physiological	Suda *et al.*, 1988

Table 1.2; Examples of acute stressors that induce upregulation of AVP mRNA in the rat hypothalamus.

Stress Type	Stress Category	Reference (s)
Immobilisation	Psychological	Bartanusz *et al.*, 1993*a*, *b*, 1994; Makino *et al.*, 1995
Restraint	Psychological	Harbuz *et al.*, 1994; Herman, 1995
Hypertonic saline injection	Physiological	Lightman & Young, 1988; Harbuz *et al.*, 1994

The response to chronic (i.e. repeated) stress is much more complex and involves differential changes in CRH and AVP mRNAs and the number of CRH/AVP co-localising neurones. It is in this context AVP appears to play a more central role in the stress response. Depending on the chronic stimulus used CRH mRNA in the parvocellular sub-division of the PVN may increase, decrease, or remain unchanged (see Table 1.3). Where habituation occurs it appears to be stressor specific as though there is no longer a response to the same stressful stimuli, alternative acute stressors may still be

Table 1.3; CRH mRNA responses in the rat PVN following chronic stress. The response of CRH mRNA following stress, as compared to controls, is indicated by arrows where; ⇧ = an increase, ⇩ = a decrease, ⇨ = no change.

Stress Type (& category)	CRH mRNA response	Reference
Immobilisation (Psychological)	⇧	Mamalaki, 1992, Bartanusz *et al.*, 1993*b*, Makino *et al.*, 1995, Gomez *et al.*, 1996
Hypertonic saline injection (Physiological)	⇧	Lightman & Young, 1989
Immobilisation (Psychological)	⇨	Ma *et al.*, 1997*a*
Adjuvant arthritis (Physiological)	⇩	Harbuz *et al.*, 1992

able to elicit a normal stress response (Kant *et al.*, 1985; Spencer & McEwan, 1990). AVP appears to play a key function in the maintenance of ACTH release in such instances. Indeed, there is evidence to suggest that AVP plays an important role in maintaining HPA axis sensitivity during repeated or chronic stress (De Goeij *et al.*, 1991, 1992; Scaccianoce *et al.*, 1991; Harbuz *et al.*, 1992). For example, after repeated restraint there is a transcript-specific alteration in gene regulation within the parvocellular PVN neurones in which CRH gene transcription declines (Ma *et al.*, 1997*a*) with a concurrent loss of CRH receptors on pituitary corticotropes (Hauger *et al.*, 1988, 1990). However, AVP gene transcription is maintained (Ma *et al.*, 1997*a*) with a sensitisation of ACTH producing cells to AVP (Hashimoto *et al.*, 1988). Furthermore, exogenous CRH has no effect on plasma ACTH levels in chronically restrained rats though exogenous AVP increases plasma concentrations of both ACTH and corticosterone (Hashimoto *et al.*, 1988). During repeated restraint stress, increases in AVP stores and increased co-localisation with irCRH neurones occurs (DeGoeij *et al.*, 1991), presumably reflecting the elevated levels of AVP required for the maintenance of appropriate levels of ACTH secretion upon renewed demand (Dallman, 1993).

In fish, information on the effects of stress on CRH and AVT secretion is scarce. Olivereau and Olivereau (1991*a*) noted an increase in cross-sectional area of magnocellular, but not parvocellular, CRH neurones in sham-adrenalectomised eels. In contrast, sham-hypophysectomised eels showed no such changes in CRH or AVT cell size in either parvo- or magno- neurones of the PON (Olivereau & Olivereau, 1988*b*). In the brook trout, *Salvelinus fontinalis*, whole brain AVT peptide content, as determined by radioimmunoassay, increased in fish subjected to a sub-lethal acid stress (Hontela *et al.*,1991, 1993) though the measurement includes both parvo- and magno- cellular neurones so it is unclear as to which cell type is responding.

Very few transcript studies have been carried out. In the rainbow trout transfer from freshwater (FW) to 80% seawater (SW) causes a brief significant rise in IT mRNA in the PON but a decline in AVT mRNA (Hyodo & Urano, 1991). The relative contribution of stress and osmotic challenge are difficult to determine. In a more recent study using *in situ* hybridisation, Ando and co-workers (1999) found that although no CRH transcripts were visible in the PON of unstressed rainbow trout, a very weak CRH mRNA signal

became detectable following a confinement stress. Although this demonstrates a rise in CRH mRNA, it is unclear which neuronal type was viewed. In addition, only two control animals, and three stressed fish were tested. In work on the goldfish, sham-implant surgery resulted in a transient decline in total telencephalon and preoptic region CRH mRNA (Bernier *et al.*, 1999). Again, it is unclear which neurones are responding since this work employed a Northern blot method on dissected, overlapping brain regions which included both parvo- and magno- cellular perikarya.

As can be seen, quantitative experiments on the response of AVT and CRH neurones to stress are lacking in fish. Such an understanding is important as these neuropeptides have been implicated, as in rats, in a number of behavioural and physiological responses. Thus, Larsen and co-workers (1998) showed that CRH will elicit thyrotropin stimulating hormone (TSH) release from coho salmon (*Oncorhynchus kisutch)* pituitaries *in vitro*. In the tench (*Tinca tinca*), a central role for CRH in the regulation of the thyroid gland has already been established (De Pedro *et al.*, 1995). As in the rat icv administration of CRH reduces appetite in both the tench and goldfish (De Pedro *et al.*, 1993, 1995, 1998). In the eel CRH also appears to play a role in the control of growth hormone (Rousseau *et al.*, 1999). AVT is implicated as central to the socially mediated sexual differentiation of fishes and thus may possibly control reproductive plasticity (Foran & Bass, 1998, 1999).

1.3.2 Corticosteroid negative feedback

Corticosteroids discharged in response to ACTH modulate the HPA axis response by completing a negative feedback loop at the pituitary and hypothalamic level. In the rat this is mediated via two receptor types, mineralocorticoid (MR) and glucocorticoid (GR) receptors (Harbuz & Lightman, 1992). MR activation is primarily associated with the low basal corticosterone levels that occur during the circadian trough, though they also play a role in facilitating the GR activation that transpires during the circadian peak or following acute stress (Spencer *et al.*, 1998). Variation in corticosteroid sensitivity is achieved by adjustments in the number of glucocorticoid receptors (Herman *et al.*, 1995; Young *et al.*, 1995; Kitraki *et al.*, 1999).

In the rat, this corticosteroid negative feedback effect acts at both the pituitary (Tanahill,1987) and hypothaalmic level (Lightman & Young, 1989), by modulating the

release and transcription of ACTH, CRH, and AVP (Eberwine & Roberts, 1984; Plotsky & Sawchenko, 1987; Harbuz & Lightman, 1989*a*). There also appears to be an extra-hypothalamic component to the negative feedback mechanism (Herman *et al.*, 1989).

In fish only one receptor, GR type (Ducouret *et al.*, 1995), has been identified. These are located throughout the peripheral tissue and within the brain (Chakraborti *et al.*, 1987; Pottinger, 1991; Lee *et al.*, 1992; Shrimpton & Randall, 1994; Teitsma *et al.*, 1997; Allison & Omeljaniuk, 1998). Within the brain these receptors are found throughout the hypothalamus of rainbow trout (Teitsma *et al.*, 1997). Thus the potential to respond to steroid feedback at the hypothalamic level exists in fish, as one would expect.

As in rats, cortisol appears to modulate the release of pituitary ACTH, and hypothalamic CRH and AVT in the eel (Olivereau & Olivereau, 1990*b*, 1991*a*). Furthermore, in the goldfish, CRH transcripts are reduced following cortisol treatment, and upregulated after treatment with the cortisol antagonist, RU486 (Bernier *et al.*, 1999). Cortisol negative feedback is covered in further detail in Chapter 5.

1.3.3 Modulatory effects of melanin concentrating hormone (MCH)

MCH, melanin concentrating hormone, acts as a colour change hormone in fish (Rance & Baker, 1979) by inducing melanin aggregation in the skin melanophores leading to skin pallor (Baker *et al.*, 1986). Consequently, plasma concentrations are higher in fish kept on a light background, than those kept in black tanks (Kishida *et al.*, 1988). MCH is located in two hypothalamic nuclei, the nucleus lateralis tuberis (NLT), and the LVR, or lateral ventricular recess (Baker *et al.*, 1995). Immunoreactive MCH fibres are found in both the pars intermedia, near the melanotropes, and the corticotropes of the pars distalis (Powell & Baker, 1987). Further to peripheral effects on the melanophores, MCH is also believed to modulate the stress response; this is thought to be exerted at the level of the hypothalamus (Green *et al.*, 1991), though some evidence suggests MCH may also influence pituitary ACTH secretion (Baker *et al.*, 1985*a*, 1986).

1.4 The Pituitary Corticotropes and Melanotropes

The teleostean adenohypophysis is separated into two distinct regions, the pars intermedia, which contains the melanotropes and the pars distalis, where the corticotropes are found. Pro-opiomelanocortin (POMC), is a multifunctional pre-pro hormone synthesised in both regions and generates, through proteolytic cleavage, several biologically active peptides (Eipper & Mains, 1980; Sawyer *et al.*, 1982). In the trout hormones resulting from POMC are varied including ACTH, which stimulates interrenal steroidogenesis, several variants of the colour change hormone melanocyte stimulating hormone (MSH), and β-endorphin, a potent endogenous opioid peptide in mammals (Kawauchi, 1983). These various peptides are produced by tissue specific cleaving enzymes. The corticotropes contain Furin and protein convertase 1 (PC1) which cleaves POMC to liberate ACTH, and the lesser products β-LPH (lipotropin hormone), and N-terminal fragments. The melanotropes, in contrast, also contain PC2 which cleaves ACTH into α-MSH, the major hormone released by melanotropes, and corticotropin-like intermediate peptide (CLIP), an insulin secretogogue (Seidah *et al.*, 1998).

Trout, being tetraploid, posses two genes for POMC, POMC-A and POMC-B (Kawauchi, 1983; Salbert *et al.*, 1992; Arends *et al.*, 1998). Interestingly these appear to be differentially regulated by sex steroids at the hypothalamic level in the trout (Salbert *et al.*, 1992), with POMC-B mRNA visible only in sexually mature individuals, whilst POMC-A is present in both immature and mature fish. The possibility of differential regulation at the pituitary level remains to be tested.

In the trout the corticotropic cells are found in the head kidney region, termed the interrenal tissue. Cortisol and corticosterone have been identified in trout (Idler & Truscott, 1972) with the former of these by far the major corticosteroid (Nandir & Bern, 1960, 1965). As in higher vertebrates adrenocorticotropic hormone, ACTH, is the major secretogogue regulating the synthesis and release of corticosteroids in fish (Wendelaar-Bonga, 1993; Wendelaar-Bonga *et al.*, 1995). Indeed, plasma ACTH (Sumpter *et al.*, 1986; Balm & Pottinger, 1995; Arends *et al.*, 1999) and corticotrope POMC mRNA (Suzuki *et al.*, 1997) are increased, along with circulating concentrations of cortisol as one would expect, following a variety of stressors. Although α-MSH will also induce

cortisol secretion it is very much less potent than ACTH (Rance & Baker, 1981; Lamers *et al.*, 1992). It has been speculated that α-MSH may contribute to pituitary control of corticosteroid release during stress in addition to ACTH (Lamers *et al.*, 1992; Wendelaar-Bonga *et al.*, 1995) and α-MSH secretion in trout certainly increases in response to some stresses, but declines after others. Following handling or confinement stress there is no change in plasma α-MSH though when these stressors are combined with a thermal shock there is a sharp rise in circulating α-MSH concentration (Sumpter *et al.*, 1985). Such reported rises in plasma α-MSH are believed to be a result of direct stimulation by the hypothalamus (Rand-Weaver *et al.*, 1993). The same applies in rats whereby a stressor specific stimulation of α-MSH occurs (Berkenbosch *et al.*, 1983, 1984). Further work is required to determine the possible role of pituitary α-MSH in the stress response. Urotensin-1, a member of the same peptide family as CRH (Lovejoy & Balment, 1999) may also stimulate the HPI axis at the level of the interrenal. In isolated interrenal preparations U-1 appears to stimulate cortisol secretion and interacts synergistically with ACTH to promote cortisol secretion in saltwater adapted rainbow trout (Arnold-Reed & Balment, 1994). In flounder (*Platichthys flesus*) a similar relationship is evident *in vitro* (Kelsall & Balment, 1998). In both species U-1 injection *in vivo* leads to dose dependant rises in plasma cortisol concentrations, suggesting that *in vitro* responses are of physiological relevance.

1.5 Trout Interrenal Tissue: Cortisol Control and Cortisol Function

At a metabolic level in fish cortisol acts as both a gluco- and mineralo- corticoid with actions on the metabolism of carbohydrates, protein and lipids (Vijayan *et al.*, 1997; Mommsen *et al.*, 1999), as well as having a significant role in marine adaptation (Kelsall & Balment, 1998), growth, reproduction (Mommsen *et al.*, 1999), and stress (Schreck *et al.*, 1989). In the latter of these roles the steroid function appears primarily to be to mobilise energy reserves (Wendelaar-Bonga & Lock, 1992) in order to prepare the animal for a reaction to the stressor. Following stress this generally induces hyperglycemia as a result of increases in hepatic gluconeogenesis initiated as a result of peripheral proteolysis (Chan & Woo, 1978; Freeman & Idler, 1973; Mommsen *et al.*, 1999). Thus, through protein catabolism amino acids are liberated into the plasma for metabolism (Barton *et al.*, 1987; Andersen *et al.*, 1991; Mommsen *et al.*, 1999).

In the short term the metabolic actions of cortisol are adaptive as they increase available energy for the animal to instigate an appropriate response. However, in the longer term persistently elevated cortisol results in well documented deleterious effects. Reduction in circulating lymphocyte or leukocyte numbers (Esch & Hazen, 1980; Hlaveck & Bulkley, 1980; Pickering *et al.*, 1982; Klinger *et al.*, 1983; Barton *et al.*, 1987), leucocrit (Wedemeyer *et al.*, 1983) and possibly lymphocyte function (Ellsaesser & Clem, 1986) as a result of stress or exogenous corticosteroid treatment are believed to be mediated by cortisol (Pickering, 1984; Barton *et al.*, 1987). Indeed, exogenous cortisol treatment can increase the susceptibility of fish to a variety of diseases (Robertson *et al.*, 1963; Roth, 1972; Pickering & Duston, 1982) which presumably is a function of the listed effects of cortisol on the immune system. Persistently raised plasma cortisol can also lead to a reduction in growth rate and condition factor, and lowered liver glycogen (Pickering & Duston, 1982; Davis *et al.*, 1985; Peters & Schwarzer, 1985; Barton *et al.*, 1987). Some of these effects may be due to cortisol-mediated deleterious changes in gut morphology (McBride & van Overbeeke, 1971; Peters, 1982; Willemse *et al.*, 1984).

1.6 Study Aims

Although the types of stressors which induce a cortisol response, and the subsequent deleterious effects when prolonged, have been well documented in fish it is difficult to gain an understanding of the response at the hypothalamic level by measuring circulating cortisol or plasma ACTH alone. There have been no quantitative transcript studies on either the response of the hypothalamic neuropeptides, believed to be central to the stress response (i.e. CRH, AVT, IT and possibly urotensin), or the actions of cortisol on transcription of these peptides. That these peptides may have behavioural effects in the brain, as in rats, makes their regulation of particular interest. Furthermore, much of the work on MCH's function in the modulation of the stress response in fish is based on circumstantial evidence and requires more detailed investigation.

This thesis describes an investigation into the responses of hypothalamic parvo- and magno- cellular AVT and IT transcripts and pituitary POMC mRNA (ACTH and MSH) to a variety of acute and chronic stresses in the rainbow trout, *Oncorhynchus mykiss*. For the purposes of this work a quantitative *in situ* hybridisation method was developed for both AVT and IT. Although a probe to salmon CRH had been successfully applied to

rainbow trout brain tissue by other workers (Ando *et al.*, 1999), this could not be consistently repeated in the current study. Consequently a reliable, repeatable *in situ* hybridisation method, using probes to the salmon CRH sequence, was not achieved despite numerous attempts. The consequence of exogenous cortisol on AVT and POMC transcription was also examined to try and clarify the cortisol negative feedback effect on these mRNAs. Finally, the response of MCH mRNA to stress and its possible modulatory effect on hypothalamic transcript levels and the HPI axis was investigated.

Chapter 2

General Materials and Methods

2.1 Fish Husbandry

All female rainbow trout (*Oncorhynchus mykiss*) eggs were reared in our aquaria from eyed eggs obtained from Exmoor Trout (North Molten, Devon, UK) approximately 18 days post-fertilisation. Fish were reared in black or white raceways fed by well aerated, UV sterilised, running well water at a rate of $1.5Lmin^{-1}$. Temperature was maintained at 11±1°C on a long photoperiod (16L:8D) with lights on at 06.00h. Development varied slightly between year classes but typically followed the course shown in Table 2.1.

Table 2.1: Timing of physiological events during fry development.

Age	Developmental Event
-22 days	Eggs fertilised at Exmoor Trout
-4 days	Eggs arrive (late Dec./early Jan.)
0 days	Eggs hatch
10 days	Fish respond to background colour
17 days	Yolk sac largely absorbed, first feed given
6 months	Transferred to 450L stock tanks
5 months	Acclimated to tap water over seven days

Feed was graded, beginning with a commercial powdered feed given at 3% body weight per day over three meals (09.00h, 12.30h, and 17.30h). This was gradually increased in size until a pelleted feed was accepted. Typically this was when fish reached 40g in weight. At this point fish were transferred to 450L stock tanks and the feeding rate gradually reduced to 1% body weight per day given in a single meal between 09.00h and 09.30h. All feed was manufactured by BOMC Paul Ltd. (Fish Food Group, Renfrew, UK). Fish were kept on the same coloured background throughout their life. For the purposes of stress experiments fish reared in white tanks were used unless otherwise indicated.

Water quality in 450L tanks was maintained with a flow-through of tap water ($1Lmin.^{-1}$) and periodic partial water changes. Uneaten feed and faeces was collected in traps at the bottom of each tank. These were emptied on a daily basis. Stocking density in holding tanks was 19 – 28 g/L. There were no disease problems over the course of this study.

2.2 Stress Paradigms

Confinement was used as the primary means of stressing fish as it successfully raises plasma cortisol titres, is simple to apply, does not disturb other fish in the aquarium, and has been shown to be a suitable means of stimulating CRH and AVP gene expression in the rat (Ma *et al.*, 1997*b*). Confinement is also deemed to be one of the few standard stress protocols in fish, and results in highly reproducible HPI axis activation (Sumpter *et al.*, 1986; Pottinger & Pickering, 1992). Furthermore, it has been widely used to study the effects of stress on a number of different aspects of fish physiology (e.g. Sumpter *et al.*, 1985, 1986; Pickering & Pottinger, 1987*a*, *b*; Pottinger *et al.*, 1992; Pottinger & Morgan, 1993; Pottinger *et al.*, 1995; Barton *et al.*, 1998; Quabius *et al.*, 1997; Vijayan *et al.*, 1997; Morehead, 1998; Ando *et al.*, 1999; Arends *et al.*, 1999; Micale *et al.*, 1999; Nolan *et al.*, 1999; Pottinger & Carrick, 1999; Ruane *et al.*, 1999; Hobby *et al.*, 2000).

Details on the application of confinement stress, and that of other stressors, are given in further detail in later chapters.

2.3 Collection of Plasma

At autopsy fish were deeply anaesthetised in 0.06% phenoxyethanol (Sigma Chemical Co., Poole, Dorset, UK) and the weight recorded. A 1-3ml blood sample was then collected from the severed caudal vessels into chilled tubes containing either 25µl (fish weight 60-150g), or 50µl (fish weight +150g) 6% Na_2EDTA as anticoagulant. The samples were centrifuged at 4000rpm for 15 minutes (4°C) and the plasma supernatant collected and stored at -20°C until required.

2.4 Collection of Brains and Pituitaries

Following decapitation, the brain was exposed and 500µl of 4% PFA (paraformaldeyhyde - see Appendix) fixative injected into the ventricles via the optic tecta. The brain and pituitary were then removed and post-fixed in PFA for 18-24 hr. at 4°C. The process following this varied depending on whether the tissue was to be wax embedded or frozen for cryo-sectioning.

2.4.1 Wax embedded tissue

Once fixed, tissues were washed in 6-8 hourly changes of distilled, de-ionised, filtered water (MilliQ water; Millipore, Bedford, MA, USA), then left in 70% alcohol at 4°C overnight. Following this brains and pituitaries were dehydrated in graded alcohols; 90% alcohol (1 hr.), 100% alcohol (1 hr.), 100% alcohol (30 mins.) and, 100% alcohol (30 mins.). Pituitaries were eosin stained (0.02% eosin in 100% alcohol) during the final 15 minutes of the last absolute alcohol wash. The pars intermedia and pars distalis stain differentially with eosin allowing identification of the two regions and thus correct alignment of pituitaries when wax embedding. Once dehydrated tissue was cleared in two xylene changes of an hour each. Wax permeation was carried out at 60°C in a bench top oven (Townson and Merce Ltd., Croydon, London) using Lambwax W/1 with added polymers (melting point, 57-58°C; Raymond A. Lamb, London). The duration of this stage varied with tissue type; pituitaries were left for 45 minutes whilst brains were left for one and a half hours.

Once permeated, tissue was embedded in wax filled paper boxes, each with a clearly marked pencil line along the bottom. This transferred to the wax once set and allowed consistent orientation of tissue prior to cutting. Brains were arranged such that the pencil line dissected the mid-line, dorsal side down. Pituitaries were orientated in a similar fashion with the pencil line running through the mid-line; the pars distalis being distinguishable from the pars intermedia by virtue of its lighter eosin staining. Once embedded the wax blocks were left undisturbed overnight at room temperature to harden.

Serial transverse sections were cut on a Spencer AO 820 microtome (Spencer, Buffalo, New York, USA) and mounted in pairs on duplicate (hypothalamus: 10 μm) or triplicate (hypothalamus: 7 μm; pituitary: 10 μm) gelatinised slides. Details of the brain region collected is covered in more detail in Section 2.5.6. Once collected slides were dried at 37°C for 24 hours prior to *in situ* hybridisation.

2.4.2 Frozen tissue

Brains to be used for cryo-sectioning were dissected out, snap frozen unfixed on crushed

dry ice, and stored at -40°C until required. Frozen section cutting was carried out on a 5030 cryostat microtome (Bright Instruments Ltd., Huntingdon, UK). Prior to sectioning tissue was placed into the microtome chamber for approximately 30 minutes to allow it to reach -15°C. Once equilibrated brains were mounted onto a sterile metal chuck using OCT compound (BDH, Lutterworth, Leicestershire, UK). Serial transverse sections of 15μm were cut and mounted in pairs on duplicate sets of gelatinised slides. Following two minutes of drying on a hand-warm hotplate slides were returned to -15°C for five to ten minutes. Sectioned tissue was stored at -80°C until *in situ* hybridisation.

2.5 *In situ* Hybridisation

2.5.1 Chromic-gelatine slide coating

The following were weighed out and wrapped in foil until required - 2.25g gelatine (Sigma) and 0.23g chromic potassium sulphate (Sigma). The gelatine was then slowly added to 800mls of MilliQ water at 70°C until fully dissolved. Once cooled to 4°C the chromic potassium sulphate was gradually added and mixed in with gentle stirring. The resulting chromic-gelatine subbing solution was topped up to 900mls with MilliQ water, covered, and stored at 4°C for a maximum of three weeks prior to use.

Pre-washed, twin frosted microscope slides (H.V.Skan, Solihull, West Midlands, UK) were soaked in 0.25% Teepol (BDH) and lukewarm water for one hour. Following a 15 minute wash in flowing tap water slides were run through eight rapid distilled water rinses, and two minutes in the chromic-gelatine subbing solution. After drying overnight the slides were given a further minute in the subbing solution. A further 12 hours of drying completed the process upon which slides were stored at room temperature in dust proof boxes.

2.5.2 ^{35}S end-labeling of oligodeoxyribonucleotide probes

The following were placed in the given order into a sterile Eppendorf tube and vortexed briefly;

28µl DEPC treated MilliQ water

10µl 5x TdT buffer (Roche Diagnostics, Sussex, UK)

5µl Cobalt Chloride (Roche Diagnostics)

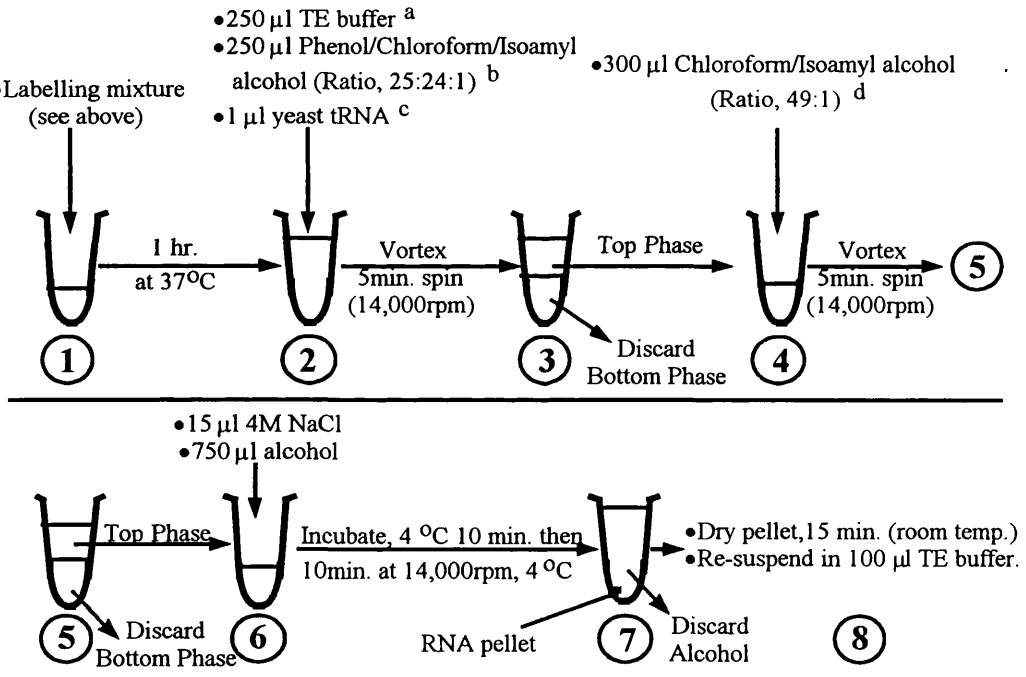
1µl 5µM oligodeoxyribonucleotide probe (Perkin Elmer, Chesire, UK)

5µl ^{35}S-α-dATP (NEN Du Pont, Stevenage, UK)

1µl TdT (Roche Diagnostics)

The tube was spun briefly, and then incubated at 37°C for one hour. The labelled probe was purified using the phenol-chloroform extraction method as shown (Figure 2.1). Following re-suspension a 1µl aliquot of the labelled probe was measured, in duplicate, in 5mls of Optiphase Safe scintillation fluid (Fisons Chemicals, Loughborough, Leicestershire, UK) in order to check its activity. The counts per minute (CPM) per aliquot were recorded for later use.

Figure 2.1: Phenol-chloroform method for the extraction and purification of ^{35}S end-labelled oligodeoxynucleotide probes.



Notes:
a) TE Buffer (pH8); 1ml of 1M Tris pH8 + 200 µl EDTA-disodium salt + 800ml MilliQ water. Tris (pH8); 8.88g Trisma HCl (Sigma) + 5.3g Trizma Base (Sigma) + 100ml MilliQ water.
b) Phenol/Chloroform/Isoamyl alcohol (Sigma).
c) Yeast tRNA (Sigma); 25mg/ml.
d) Chloroform (Fisher Scientific UK Ltd.); Isoamyl alcohol (Sigma).

2.5.3 Pre-hybridisation techniques

Due to the susceptibility of target mRNAs to degradation by RNAses every effort was made to keep all pre-hybridisation solutions, glassware, and plasticware RNAse free. Solutions in these steps were treated overnight with 1ml/L of diethylpyrocarbonate (DEPC; from Sigma). This was followed by autoclaving to denature the DEPC. Plasticware was left overnight to soak in a 0.1% solution of DEPC in MilliQ water prior to autoclaving. Glassware was rendered RNAse free by baking at 200°C overnight.

For wax embedded tissues the pre-dried slides were dewaxed and rehydrated through a series of xylene and graded alcohols thus; xylene (x3, 10 mins. each), 100% alcohol (x2, 5 mins. each), 90% alcohol (5 mins.), 70% alcohol (5 mins.), MilliQ water (x2, 5 mins. each). Following this slides were equilibrated in 2x sodium saline citrate (SSC – see Appendix) at 60°C (10 mins.) then transferred to fresh MilliQ water (room temperature).

In the case of frozen tissue slides were left to thaw on a sheet of foil for ten minutes prior to a five minute fixation in 4% PFA (pH 7.2) at room temperature. Sections were washed for ten minutes in 0.1M phosphate buffered saline, PBS (pH7.2), followed by ten minutes in 1.4% 0.1M triethanolamine (Sigma) and 0.25% acetic anhydride (Sigma) in 0.9% saline (Baxter Health Care, Thetford, Norfolk, UK). A series of graded alcohols and a chloroform step served to dehydrate and de-lipidate tissue as follows; 70% alcohol (1 min.), 80% alcohol (1 min.), 95% alcohol (1 min.), 100% alcohol (1 min.), 100% chloroform (5 mins.), 100% alcohol (1 min.), and 95% alcohol (1 min.). Slides were left to air dry at room temperature prior to hybridisation.

2.5.4 Hybridisation and post-hybridisation washing

This process is common to both wax embedded and frozen sections. For the hybridisation reaction, 100µl of hybridisation mixture (see Appendix) containing ^{35}S-labelled oligoprobe, at variable final activity (see Table 2.2), was added to each slide ensuring all sections were covered. This was coverslipped with a rectangle of Nescofilm (BDH) and incubated in a humid chamber at 37°C overnight. All oligoprobes were synthesised by Perkin Elmer.

After hybridisation, sections were given four vigorous rinses in 1xSSC at room temperature to remove the coverslip and any excess hybridisation buffer. This was followed by four washes for 15 minutes each in 1 xSSC at a temperature specific to the melting temperature of the oligoprobe being used (see Table 2.2). Two final rinses in 1x SSC (room temperature) were followed by a brief rinse in distilled water. Slides were thoroughly dried in an incubator at 37°C.

2.5.5 Autoradiography

Once dried slides were arranged in 30x40cm Hypercassettes (Amersham International Plc., Buckinghamshire, UK) and opposed to autoradiographic film along with low and

Table 2.2: Sequence, washing temperature, and final activity of routinely used oligodeoxyribonucleotide probes. CPM/100µl is the final CPM of the given probe in 100µl of hybridisation mixture. AVT = Arginine Vasotocin, IT = Isotocin, MCH = Melanin Concentrating Hormone, POMC = Pro-opiomelanocortin. Com = a sequence that encodes for regions common to both variants of the gene. Washing temperature is quoted in °C.

Probe Name	Probe Sequence (5'–3')	CPM /100µl	Washing Temp.
Salmon AVT	tt ccc gcc tcg cgg aca gtt ctg gat gta gca cgc aga gga gag cg	$6x10^4$	60
Salmon IT	tt gcc tcc gat ggg gca gtt gga gat gta gca ggc tgt gca gac ag	$6x10^4$	60
MCH-Com	tcc cac cat gca cct cat ggt gtc	$3x10^5$	55
POMC-A	ggc tct aag tcc tac agt gac att	$5x10^4$	60
POMC-B	agt ttt caa cag ttg cca ctg gtc t	$5x10^4$	60
POMC-Com	ccg gcg gaa gtg (c/t) tcc atg gag tag ga	$1x10^5$	60

high ^{14}C microscales (Amersham). These are calibrated for the auto-absorptive features of intact brain matter with a tissue equivalent range of 0.14 - 127.2 nCi/g brain tissue (low), and 39.4 - 1075.1 nCi/g brain tissue (high). Exposure times were selected so that tissue with the weakest signal gave an image easily distinguishable from background whilst the most radioactive were not exposed beyond the films' upper limit.

Consequently exposure duration varied dependent on the probe, tissue, and cell type. Typical times are shown in Table 2.3.

Once exposed films were developed by hand with Photosol CD18 X-ray developer (Photosol Ltd., Basildon, Essex, UK), rinsed in tap water, and fixed with Kodak Unifix (Sigma) for five minutes each. A 15-20 minute rinse in running tap water removed excess fix. All these stages were carried out under safelight illumination. A final wash with 2-3 drops of Kodak Photoflo (Sigma) in distilled water was carried out before films were hung up to dry.

Table 2.3: Typical autoradiographic film exposure times for commonly used oligodeoxyribonucleotide probes. Note that for POMC B in the corticotropes an exposure time of eight weeks failed to show any visible signal. For AVT and IT the exposure time given applies to both parvo- and magno-cellular neurones. NLT = nucleus lateralis tuberis, LVR = lateral ventricular recess.

Probe Name (cell type)	Exposure Time
Salmon AVT	3 days
Salmon IT	3 days
MCH Common (NLT)	12-18 hours
MCH Common (LVR)	7 days
POMC A (Corticotropes)	7 days
POMC A (Melanotropes)	2 days
POMC B (Corticotropes)	No signal
POMC B (Melanotropes)	2 days
POMC Common (Corticotropes)	7 days
POMC Common (Melanotropes)	24 hours

In order to allow photography of positive signals radio-labelled slides were dipped in photographic emulsion and developed. All stages were carried out under safelight. To 6mls of MilliQ, 30µl of a 50% glycerol solution (in MilliQ) was added. This was made up to 10mls of dipping solution by the addition of nuclear research emulsion (Ilford, UK), and thoroughly mixed. Each slide was placed into the emulsion for approximately 10 seconds, drained, and then left to dry in a light-proof box. Dipped slides remained, with

desiccant, in these boxes for a probe-dependent exposure time. Typically this was determined as three-times the required exposure time for autoradiographic film. Thus, for example, dipped slides would be exposed for nine days where film exposure was three days. Following exposure, slides were placed into developer (Kodak D-19, Sigma) for 3.5mins, a stop bath of MilliQ water for 30 seconds, and then fix (Kodak Unifix, Sigma) for 3.5 mins. Following five minutes of washing under running tap water slides were dehydrated in graded alcohols, cleared in xylene, and mounted in DPX.

2.5.6 Analysis of *in situ* hybridisation

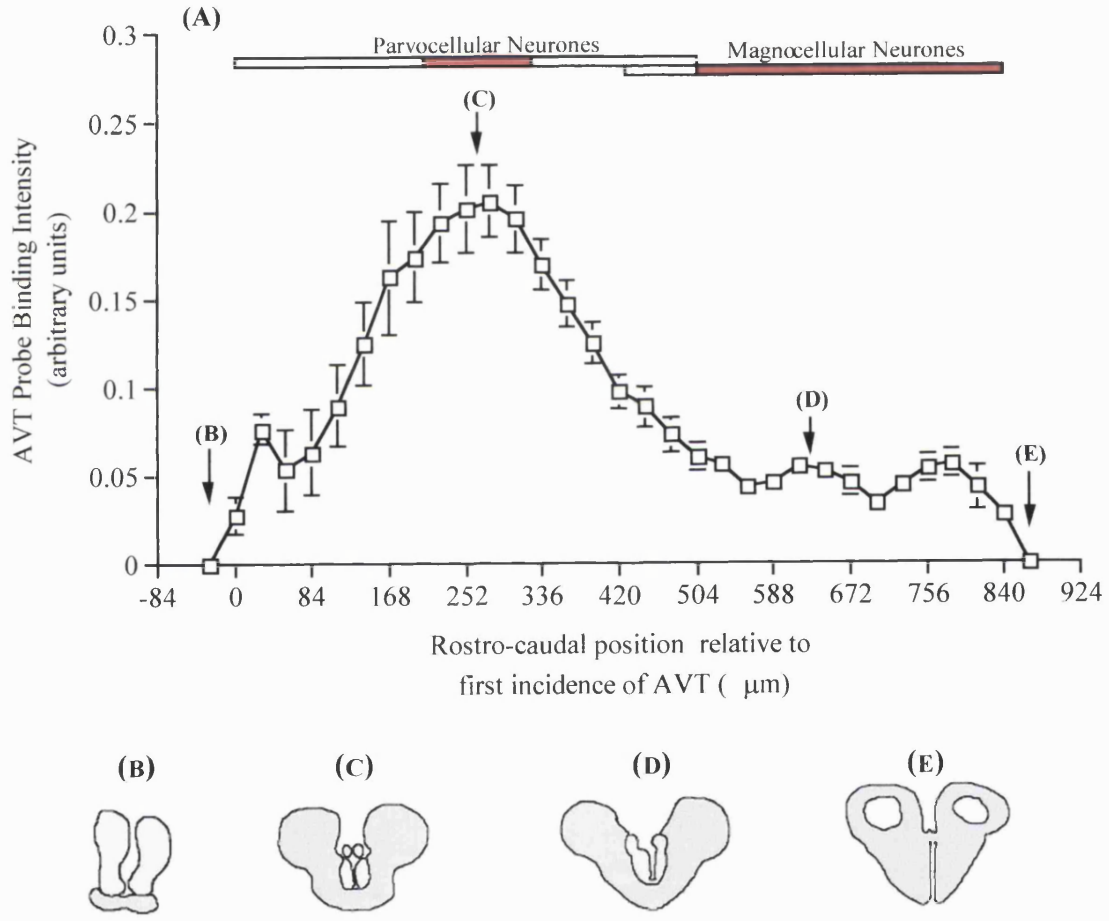
The autoradiographic signal was quantified using a computer densitometry system. For this an Apple Macintosh IIci computer running 'NIH Image' connected to a high magnification video camera was used to capture images of the autoradiographic film. The ^{14}C standards were used to construct a standard curve (third degree polynomial) of signal intensity versus radioactivity. This also accounted for the non-linear response of film to β-irradiation. A threshold tool and image capture system removed the contribution of the background from that of the hybridisation signal. This also ensured that the exact limits of the signal area were determined independently of the operator. From this an optical density (OD) and signal area (A) value were obtained. The number of sections and region scanned varied with probe and cell type. The methodology used for MCH has been described previously (Francis, 1996). Since probe binding data output is in arbitrary units, and consequently prone to absolute value variations between experiments, all data are expressed as a percentage of the relevant experimental control to allow easier comparison.

No quantitative *in situ* hybridisation work has been previously carried out for AVT or IT in trout, thus the sectioning and scanning techniques used for these requires more detailed explanation.

Once fixed, trout brains were wax embedded and the entire hypothalamic region cut. The ensuing wax ribbon was carefully studied and sections defining the anterior and posterior limits of the AVT and IT neurones identified. The anterior limit is marked by the start of the pre-optic recess (see Figure 2.2B). Sections were collected from this point through

the pre-optic nucleus until the nucleus lateralis tuberis (NLT) was clearly visible (see Figure 2.2E). This area encompasses both parvo- and magno- cellular neurones.

Figure 2.2; Distribution and intensity of arginine vasotocin (AVT) messenger ribonucleic acid (mRNA) in the rainbow trout brain. (A) Intensity of AVT hybridisation signal (arbitrary units) at different rostro-caudal levels of the pre-optic nucleus of unstressed rainbow trout. Each value is the mean of 23 fish (±SEM); the value for each fish was the average from paired sections at each level. The bar across the top indicates the cell types found at each level. Regions used for scanning purposes are marked by red shading. (B), (C), (D) and (E) are illustrations of typical transverse sections of rainbow trout brain at the indicated points.



Parvocellular AVT and IT neurones cover a large number of sections; this, coupled with the size of experiments, meant that all sections in this region could not be scanned. Consequently a preliminary analysis was carried out on sample fish taken from the first experiment to design a suitable, repeatable scanning method. Three different

permutations were tried:

(A) Alternate pairs

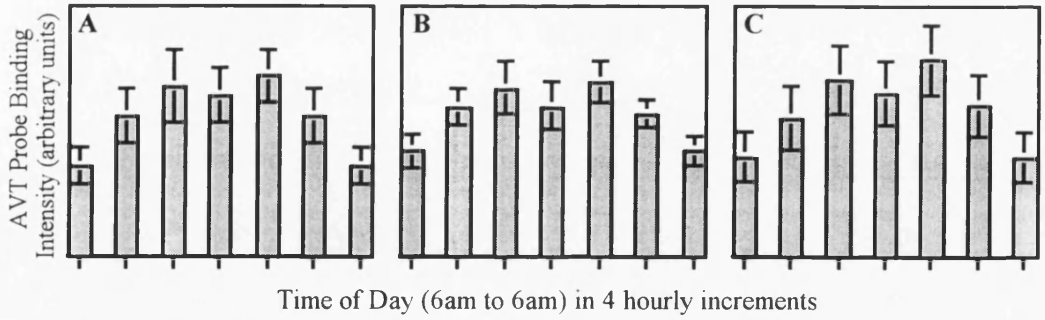
(B) Central six consecutive sections

(C) Every 4th pair

The optical density (OD) and signal area (A) values were used to calculate probe binding intensity (ODxA) per section. The mean per fish and per treatment was subsequently calculated from this. In all cases the posterior limit of the parvo-region was excluded since this overlaps with magnocellular neurones. Results are shown in Figure 2.3.

All methods used produced the same trends. Ultimately, however, Method B was chosen. This method clearly identified trends expressed by the whole, and covered the largest proportion of parvocellular neurones in the shortest processing time. Method B consisted of selecting six paired sections/brain on the basis of location, mid-way along the parvocellular mass (see Figure 2.2A). For magnocellular neurones, since the signal area and number of sections they cover is less than that of parvocellular perikarya, all sections could be scanned. Again, the overlap area was not scanned. Probe binding intensity per section and per treatment was calculated as for parvo-neurones.

Figure 2.3; AVT probe binding intensity in the parvocellular region; results derived from three different scanning methods. (A) Alternate pairs of sections, (B) a representative of six sections from the mid-parvocellular region, and (C) every 4th section scanned. Bars are means (±SEM). n=3 per point.



2.6 Plasma Cortisol Radioimmunoassay

A 100µl aliquot of plasma was dispensed into a 4 ml polypropylene tube to which 500µl

of absolute alcohol was added. After mixing another 500µl of 100% alcohol was added. Following further mixing tubes were centrifuged for 15 minutes (4000rpm at 4°C). Appropriate samples of the supernatant (50-400µl depending on expected final cortisol concentration) were taken in duplicate and transferred to new polypropylene tubes. Alongside this 10µl aliquots of cortisol standards (12.5pg-1600pg/10µl; see Section 2.6.1) were added, in duplicate, to polypropylene tubes. These were all dried under vacuum until no liquid remained. Typically this took between one and three hours depending on the sample volume.. Contents were re-suspended in 200µl of a prepared PBSG (0.05M PBS, pH 7.5, with 0.1% gelatine) containing ^{3}H-cortisol and cortisol antiserum (see Section 2.6.2). The tubes were covered and left to be incubated overnight at 4°C.

Following overnight incubation 500µl of ice cold dextran charcoal solution (0.125% Dextran (Sigma) and 0.5% charcoal (Sigma) in PBSG) was added to each tube. Following a 15 minute incubation samples were centrifuged for 15 minutes (4000rpm at 4°C). The supernatant was carefully decanted into scintillation vials containing 6mls of scintillant. Samples were counted on a scintillation counter and the cortisol concentration of samples calculated by reference to a standard curve.

2.6.1 Cortisol standards

To 20mls of absolute alcohol, 3.2mg hydrocortisone (Sigma) was added to create a cortisol stock solution (1.6µg/10µl). Working standards (1600pg/10µl down to 12.5pg/10µl) were prepared from this by serial dilution in absolute alcohol. Standards were stored at –20°C until required.

2.6.2 ^{3}H-cortisol and cortisol antiserum preparation

The tritiated cortisol (^{3}H - (1,2,6,7) - cortisol, 250µCi in 250µl toluene/alcohol, 9:1 from Amersham) was diluted in 25ml of toluene/alcohol (9:1) and kept at –20°C. Cortisol antiserum (Cortisol R5, raised by Dr.Gilham, University of Bath) was stored at 4°C at x10 dilution in PBSG (0.05M, pH 7.5). For use a ^{3}H-cortisol/PBSG/cortisol antiserum (400:2:1) solution was made. Absolute volumes were calculated to allow sufficient for

200µl per assay tube. To prepare the solution the ^{3}H-cortisol was dried down in a glass beaker, then re-suspended in PBSG buffer. The cortisol antiserum was added to this immediately prior to use. A 200µl aliquot of this mixture was added to each assay tube.

2.7 Arginine vasotocin radioimmunoassay

The AVT radioimmunoassay was carried out on pituitary and hypothalamus tissue. Tissue was dissected out, placed into 500µl of ice cold 0.1M HCl and sonicated at 4°C. Homogenised tissue was stored at -20°C until assay.

Throughout the procedure a solution of 0.05M PBS with 0.05M EDTA and 1% Bovine Serum Albumen (BSA), pH7.6 was used as the assay buffer. The AVT antibody (PMI 83; kindly donated by Pat Ingleton) was stored at 4°C as a 0.6% solution in assay buffer until needed. Standards were created from AVT peptide (Sigma) in assay buffer and ranged from 15 to 4000pg/100µl. All steps were carried out on ice when possible. For the radioimmunoassay 100µl of the sample, or a standard (15-4000pg), was placed into an Eppendorf tube along with 50µl of AVT antibody and 50µl of ^{125}I-AVT (see Section 2.7.1). A non-specific binding tube was also created in which the AVT antibody was replaced by a further 50µl of assay buffer. Following through mixing and a brief centrifuge tubes were incubated for two days at 4°C.

Bound and free AVT were separated by the addition of 500µl of Dextran-coated charcoal as described previously for plasma cortisol (see Section 2.6). AVT peptide concentration in the sample was calculated by reference to the standard curve.

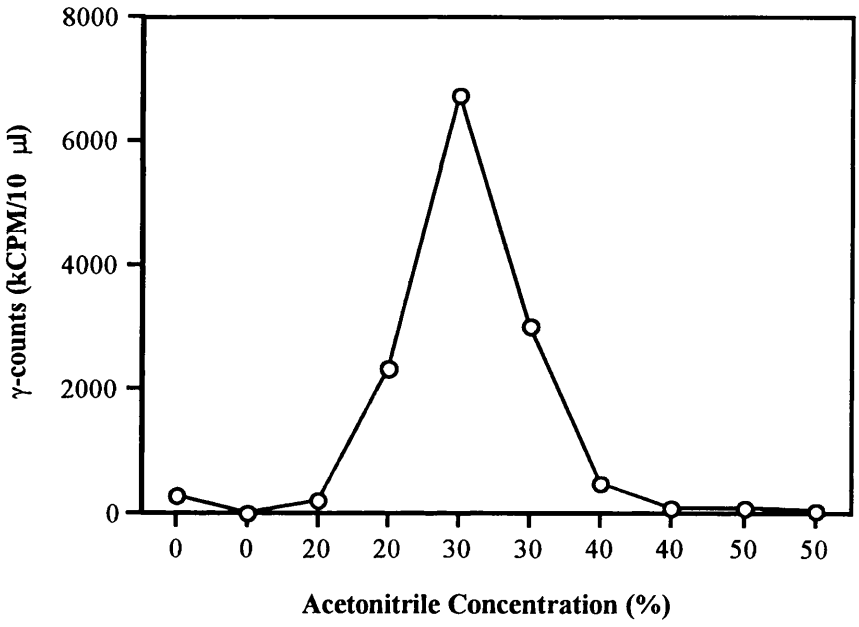
2.7.1 ^{125}I end-labeling of AVT peptide (Chloramine-T method)

A C_{18} Sep-Pak cartridge (Millipore) was prepared by washing with 2ml of the following solutions in the given order;

0.1%	tri-fluroacetate (TFA; Sigma) in distilled water
50%	acetonitrile (ACN; Sigma) in 0.1% TFA
1%	Polypep (Sigma) in distilled water
50%	ACN in 0.1% TFA
0.1%	TFA in distilled water

Arginine vasotocin peptide (20% solution with 20% Chloramine T in 0.25M PBS, pH7.4) was mixed by inversion with 1μC of ^{125}I in an eppendorf tube. After 30 seconds 600μl of a stop solution was added (0.1% mercaptoethanol (Sigma) and 0.25% Bovine Serum Albumin, BSA (Sigma) in 0.5M PBS, pH7.4). The resulting mixture was passed twice through the prepared Sep-Pak, followed by two washes with distilled water. ^{125}I-labelled AVT was washed from the Sep-Pak with graded, duplicate 1ml washes of ACN in 0.1% TFA. These began with a 20% concentration of ACN, rising in 10% increments to a 50% concentration. A 10μl aliquot of the resulting elutes was measured on a γ-counter and an elution curve plotted. The elute with the highest counts denoted that which contained the ^{125}I-labelled AVT; typically this was the first or second 30% ACN wash (see Figure 2.4). This was diluted in assay buffer to give 5000cpm per 50μl aliquot. These were stored at -20°C until use.

Figure 2.4: Typical radioactivity (kCPM/10μl) of acetonitrile elutes of a Sep-Pak containing ^{125}I end-labelled AVT peptide.



2.8 Immunocytochemistry

Brains and pituitaries to be used for immunocytochemistry were fixed in sublimated Bouin-Holland (see Appendix) for five to seven days at room temperature and embedded

in wax as described previously (see Section 2.4.1). All slides used for immunocytochemistry were washed in absolute alcohol, dried and then wiped with 15µl of glycerine albumen solution immediately prior to use. Serial sections of 5µm were cut and mounted onto these coated slides. Immunoreactivity for AVT and CRH was demonstrated using the biotin-streptavidin method with a commercial kit (Vectastain® Elite ABC Kit; Vector Laboratories, California, USA). Sections were dewaxed and re-hydrated and mercuric chloride crystals removed with an additional two steps (5 mins. in 2% iodine and 3% potassium iodide in 70% alcohol, followed by brief immersion in 3% sodium metabisulphite until tissue decolourised). Slides were transferred to 0.05M PBS (pH7.6) after five minutes rinsing in flowing tap water. Sections were incubated first for 30 minutes in 3% goat serum in 0.05M PBS, then overnight in humid chambers with primary antibody (in PBS with 3% goat serum as blocking agent). The AVT and CRH antiserums were used at a 1:1000 dilution. Following three 15 minute washes in PBS, sections were incubated for 30 minutes in biotinylated secondary antibody solution. A subsequent wash in PBS preceded a final 30 minutes in Vectastain® Elite ABC Reagent. The reaction was stopped with a PBS rinse. Slides were then transferred to 0.05M Tris HCl buffer (pH 7.5). Immunoreactiviy was visualised by soaking in 0.025% diaminobenzadine (DAB) solution in Tris HCl. Development of DAB staining was terminated with a ten second Tris HCl rinse. Slides were then dehydrated, cleared and mounted in DPX (BDH). All procedures were carried out at room temperature.

2.9 Statistical Analysis

Results were analysed using one- or two- way ANOVA, as appropriate, on log transformed data. When this indicated a statistical significance ($P<0.05$), Fisher's LSD test was applied in order to assign superscripts. In some instances a χ^2 or Mann-Whitney's U-test were used to ascertain if any experimental differences were present.

Chapter 3

Diurnal Expression of AVT and POMC Transcripts

3.1 Introduction

Most hormones display a rhythmic pattern of release. Although the reasons for this remain unclear it is believed to aid the maintenance of homeostasis by coupling behaviour and physiology with external daily events, such as the light:dark cycle. As well as responding to stress, plasma corticosteroids display such a rhythm, characterised in mammals by maximal levels just before the onset of the activity period (Krieger, 1975; Krieger & Luria, 1977). Thus, in nocturnal animals such as rats, this occurs just prior to the start of the dark phase (Kwak *et al.*, 1993). In humans, since they are active during the light period, cortisol reaches a peak just before rising, with significantly lower levels later in the day (Watabe *et al.*, 1987). In trout the peak occurs in the middle of the night (Rance *et al.*, 1982; Pickering & Pottinger, 1983). This pre-activity peak is thought to prepare the animal for the impending subjective day by virtue of the effects cortisol has on metabolic function, such as enzyme induction and the stimulation of gluconeogenesis. This rhythm can also be entrained to other cues that are of significance to homeostasis. In rats, for example, the time of feed presentation has been shown to be a potent synchroniser of biological rhythms (Krieger & Hauser, 1978) and the peak in cortisol secretion can, indeed, be manipulated by shifting the time of a single daily feed (Wilkinson *et al.*, 1979). Trout kept under aquarium conditions display a difference from that seen in the field whereby a second peak in plasma cortisol concentration is evident in the early morning (Rance *et al.*, 1982). This is likely to be due to feeding or some other activity in the aquarium that occurs at this time. It has also been speculated that it could be a response to the rapid transition from dark to light that occurs in the aquarium at the false dawn (Rance *et al.*, 1982).

In trout, cortisol secretion by the interrenal gland is largely controlled by ACTH (Donaldson, 1981; Sumpter *et al.*, 1986; Balm *et al.*, 1994) which is released from the corticotrope cells of the adenohypophsis. These in turn are stimulated by hypothalamic CRF-41 and AVT as demonstrated *in vitro* (Baker *et al.*, 1996; Pierson *et al.*, 1996). Isotocin has been demonstrated to induce ACTH release though in the trout it is less potent than either AVT or CRF (Pierson *et al.*, 1996). Urotensin-1 may also act as an ACTH secretogogue though this, as yet, has only been demonstrated in isolated goldfish pituitary cells (Fryer *et al.*, 1985).

It is likely that a central clock influences the diurnal secretion of these neuropeptide hormones. The suprachiasmatic nuclei (SCN), lying immediately above the optic chiasma, is the best studied in mammals. The clock system is believed to be composed of a number of hierarchically organised and possibly interacting oscillators (Rietveld, 1985) but the SCN is thought to be the main regulator. Indeed there is evidence to suggest that it drives the hypothalamo-pituitary-adrenal axis in rats (Hiroshige & Sakakura, 1971; Takebe *et al.*, 1972; Krieger & Luria, 1977) and lesions of this region have been shown to abolish the steroid rhythm (Abe *et al.*, 1979; Krieger & Luria, 1977). In addition, the CRH mRNA rhythm, absent in ageing rats, can be restored by the implantation of SCN cells from a young animal (Cai *et al.*, 1997). In teleosts the SCN homologue is thought to be situated in the preoptic area (nucleus anterioris periventricularis; Peter & Gill, 1975) though its importance as a regulator of daily endocrine rhythms remains unclear. There is some speculation that the pineal is also an organiser of circadian activity in fish (Ekstrom & Meissl, 1997) as in some species it displays an endogenous rhythm of melatonin secretion *in vitro*, though the literature is somewhat contradictory. Some workers have found that pinealectomy negates the influences of restricted photoperiods on diel fluctuations in blood levels of gonadotrophin (Hontela & Peter, 1980). However, in other cases the effects of photoperiodicity do not seem to be mediated by the pineal organ (Hontela & Lederis, 1985). It seems likely that although it may act as a central clock, multiple factors influence the diurnal fluctuation of hormones in vertebrates. Cortisol negative feedback, for example, could also play a role at both the pituitary and hypothalamic level, regulating the release of its own secretogogues (Lightman & Young, 1989; Kwak *et al.*, 1993).

The enhanced release of the neuropeptides that stimulate the release of corticotropin might be expected to be compensated for by an increase in messenger RNA (mRNA) transcription, though the temporal relationship between transcription and hormone release is difficult to predict. In rats, for instance, there is a daily rise in abundance of CRH mRNA that pre-empts CRH/ACTH release by several hours (Cai *et al.*, 1997; Watts and Swanson, 1989). In the stress response this is reversed, with CRH mRNA increases following several hours after neuronal stimulation and peptide release (Ma *et al.*, 1997*a*). The difference seems to be that the increase in diurnal mRNA anticipates peptide release whilst, following a stress, transcription compensates for it.

Aims

The aim of this study was to determine if there was a diurnal pattern of gene transcription in AVT and isotocin neurones of the pre-optic region of the hypothalamus, or POMC in the corticotropes and melanotropes of the pituitary. The relationship with circulating cortisol concentrations was also investigated. The determination of these neuropeptide rhythms is essential in the planning of future experiments investigating the effect of stress on AVT transcripts since differences in mRNA between two given time sample points following a stress could well be masked by the normal diurnal fluctuations.

3.2 Published Paper

Diurnal Changes in the Expression of Genes Encoding for Arginine Vasotocin and Pituitary Pro-Opiomelanocortin in the Rainbow Trout (*Oncorhynchus mykiss*); Correlation with Changes in Plasma Hormones

Gilchriest, B. J., Tipping, D. R., Levy, A., and Baker, B. I.
Journal of Neuroendocrinology, 1998, Vol. 10, 937-943.

In this paper the majority of the work described was carried out by B.Gilchriest. *In situ* hybridisations for POMC were carried out by Dr. D.Tipping. Facilities for labelling oligonucleotide probes were provided for by A.Levy. Dr. B.I.Baker supervised the work.

Journal of Neuroendocrinology, 1998, Vol. 10, 937–943

Diurnal Changes in the Expression of Genes Encoding for Arginine Vasotocin and Pituitary Pro-opiomelanocortin in the Rainbow Trout (*Oncorhynchus mykiss*): Correlation with Changes in Plasma Hormones

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Key words: arginine vasotocin; pituitary pro-opiomelanocortin; rainbow trout; plasma cortisol; diurnal neuropeptide gene expression.

Abstract

Using quantitative *in-situ* hybridization, this study monitored diurnal changes in the abundance of the gene transcripts of two corticotropin-releasing peptides, arginine vasotocin (AVT) and isotocin in hypothalamic neurones, and of pro-opiomelanocortin (POMC) mRNA in the pituitary of the rainbow trout (*Oncorhynchus mykiss*). A significant diurnal pattern of gene expression was only displayed in the hypothalamus by the parvocellular AVT neurones of the preoptic nucleus. Abundance of AVT mRNA in these neurones was low at lights on (06.00 h), increased during the morning to reach a plateau of peak values between 14.00 h and 22.00 h, and then declined during the dark phase. This pattern was the inverse of that shown by plasma cortisol values. Changes in AVT transcript abundance are also considered in terms of the reported diurnal change in circulating AVT concentration. Pituitary and hypothalamic AVT peptide content did not change. Transcripts of both POMC genes (POMC-A and POMC-B) were monitored in pituitary corticotropes and melanotropes. Only POMC-A mRNA was detected in corticotropes where it showed no diurnal change in abundance. Transcripts of both POMC genes were found in the melanotropes, although, judging from autoradiographic intensity, POMC-A mRNA predominated. Both genes showed diurnal differences in their transcription with POMC-A mRNA showing peak values at 10.00 h and a nadir at 02.00 h, while POMC-B mRNA showed an inverse pattern. The results indicate that the two POMC genes can be independently regulated.

In fish, as in other vertebrates, cortisol secretion is not only enhanced in response to stress, but also displays a diurnal rhythm under basal, stress-free conditions. In the trout, this consists of a relatively prolonged period of raised plasma cortisol during the dark phase of the 24 h cycle, with significantly lower levels during the day (1, 2). When trout are kept under aquarium conditions, cortisol may also be raised in the early morning, perhaps in association with daily feeding or some other activity in the aquarium (1).

In the trout, cortisol secretion by the interrenal gland is largely controlled by the adrenocorticotropic hormone (ACTH) from the anterior pituitary gland the release of which, in turn, is stimulated by the hypothalamic peptides CRF-41 and arginine vasotocin (AVT) (3). Other neuropeptides such as isotocin and urotensin-I may also be involved since they are ACTH secretogogues when tested on goldfish pituitary cells (4) but their effects on the trout pituitary have not been investigated.

It seems probable that in fish, as in mammals, the diurnal secretion of cortisol and ACTH is driven by a central clock, acting on one or other of these hypophysiotropic neurones. The release of neuropeptide must be compensated for by increased synthesis but it is difficult to predict the temporal relationship between release and synthesis. In the rat, for instance, there is a daily rise in the abundance of CRF-41 messenger RNA (mRNA) that pre-empts CRF/ACTH release by several hours, and which declines several hours before the diurnal surge of corticosterone (5, 6). This is different, therefore, from what happens in response to stress when CRF mRNA is significantly raised a few hours after the stress and the release of CRF.

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The aim of the present paper was to determine whether there is a diurnal pattern of gene transcription in the neurones secreting AVT and isotocin, or in the corticotropes and melanotropes of the pituitary gland and, if so, how this is associated with the diurnal activity of the hypothalamo-pituitary-interrenal axis (HPI). This information was needed as preliminary information as to the best time of day to study stress-induced changes in neuropeptide gene expression in the rainbow trout, which is the main thrust of our current research.

Results

Immunostaining of arginine vasotocin neurones

Parvocellular AVT neuronal cell bodies (parvo-AVT) were identified in the preoptic nucleus (PON) around the preoptic recess and either side of the third ventricle, above the optic chiasma (Fig. 1B). Their position coincided with the immunoreactive (ir) CRH perikarya although the latter seemed more numerous (Fig. 1C). In a few cases, it was clear by comparing neighbouring sections that AVT and CRH were co-localized within some neurones. Magnocellular neurones, which were immunostained with both AVT and CRH antisera, were seen at the dorsal limits of the parvocellular cell mass in its most posterior location (Fig. 1E) and formed a thin column of neurones either side of the medial ventricle in the dorsal hypothalamus, extending back nearly to the level of the pituitary gland (Fig. 1F).

Arginine vasotocin mRNA

The intensity of AVT antisense oligonucleotide probe binding, measured on the X-ray film autoradiographs, is taken to reflect the abundance of mRNA. A positive signal was found in the same location as the irAVT perikarya. These locations are shown in Fig. 1(A,D).

The existence of a diurnal rhythm was investigated in two separate experiments, when fish were killed at 4 hourly intervals (experiment 1) or at 2 or 4 hourly intervals (experiment 2). Both showed the same pattern of AVT mRNA expression but, for analysis, results from each experiment were normalized and pooled. A significant diurnal change in mRNA abundance was seen only in the parvo-AVT neurones (ANOVA: $P<0.002$; Fig. 2). Values were lowest at 06.00 h at the time when lights came on. They then increased progressively until early afternoon (14.00 h) and remained raised until 22.00 h after which they declined progressively to reach their nadir at 06.00 h. The magnocellular neurones displayed no significant change in AVT mRNA abundance.

Arginine vasotocin peptide

Arginine vasotocin was measured by RIA in extracts of the hypothalamus and pituitary. The results (Fig. 3) reveal no significant change in peptide concentration in either site over the 24-h period.

Isotocin mRNA

Since isotocin and AVT neurones overlap in their distribution (7), isotocin mRNA was measured in sections immediately adjacent to those used to evaluate AVT probe binding. No significant diurnal rhythm in isotocin probe binding was observed in the parvocellular neurones. (Results not shown). Magnocellular neurones were not examined.

Pituitary gland

The prevalence of both POMC-A and POMC-B gene transcripts was investigated in the corticotropes and melanotropes. In the corticotropes, only POMC-A gene transcripts could be detected, and these showed no diurnal variation (Fig. 4). In the pars intermedia melanotropes, however, transcripts for both genes were detectable. POMC-A probes gave a very strong signal and showed a significant diurnal rhythm (ANOVA $P<0.003$ using pooled data from the two experiments). Transcripts were most abundant at 10.00 h, then declined to reach low values at 18.00 h after which they remained low until 02.00 h (Fig. 5). Transcripts for POMC-B gave a much weaker signal, requiring 9 days exposure to give a modest autoradiographic signal, compared with a strong signal from POMC-A after only 3 days exposure, using labelled probes with similar cpm/μl. The abundance of POMC-B transcripts also showed significant diurnal variation (ANOVA $P<0.001$); but, surprisingly, its pattern was the inverse of that for POMC-A, i.e. lowest values in the morning and highest values in the evening until 02.00 h. (Fig. 5).

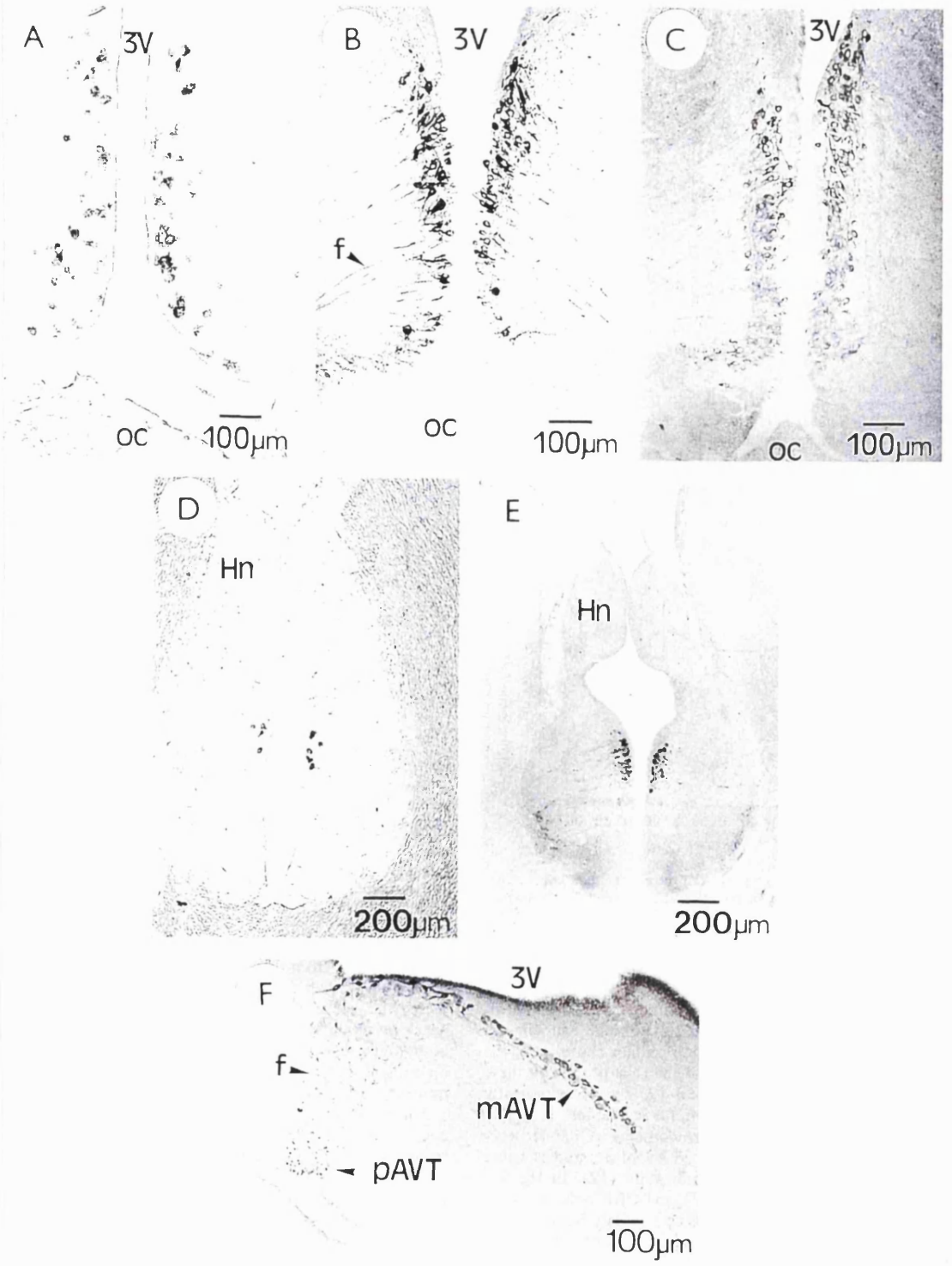
Plasma cortisol

Cortisol concentrations were low in both experiments but they nevertheless showed the expected diurnal pattern, with raised values during the night and early morning, and lowest values in the afternoon and evening (Fig. 6).

Discussion

Our immunocytochemical findings that irAVT and irCRF neurones overlap in their distribution, and that in some cases both peptides appear to occur within the same cell bodies,

FIG. 1. Sections of the trout brain illustrating the location of arginine vasotocin (AVT) and CRF peptide and AVT mRNA. (A) *In-situ* hybridization (emulsion-dipped slide) showing binding of the AVT oligoprobe to neurones located in parvocellular neurones of the preoptic nucleus (PON). The neurones are seen either side of the medial third ventricle (3V) above the optic chiasma (oc). (B) Immunostained parvocellular AVT neurones and their laterally directed fibres (f) either side of the 3V. (C) Immunoreactive CRF parvocellular neurones in the preoptic nucleus, either side of the 3V. (D, F) Magnocellular AVT neurones at the level of the habenular nucleus (Hn) identified in transverse sections by *in-situ* hybridization (D) or immunostaining (E). (F) Sagittal section of trout brain showing immunostained magnocellular AVT neurones (m AVT) lying posterior to the parvocellular AVT neurones (p AVT), whose location is identified here mainly by their fibre tracts (f). The apparent dark striations in the optic tracts of hybridized sections (A, D) are optical artifacts, arising from the illumination needed to reveal the outlines of the unstained sections. Photographs (A) and (D) are of hybridized sections that have been emulsion-dipped. They were given a long exposure to emphasize the hybridization signal. They were not used for signal quantification, which was done on X-ray autoradiographs.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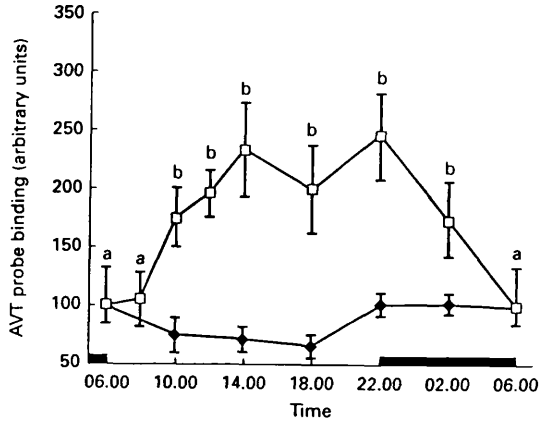

FIG. 2. Diurnal variation in the abundance of arginine vasotocin (AVT) gene transcripts in the trout hypothalamus. Values are the normalized means (±SEM) from six to 12 fish. The 06.00 h value has been repeated for clarity. The thick horizontal bars indicate the dark period. Points labelled with different superscripts (a, b) are significantly different ($P<0.05$) from each other. A significant diurnal change was seen in the parvocellular neurones (ANOVA: $P=0.002$) but not was apparent in the magnocellular neurones (ANOVA: $P=0.481$). □, parvocellular neurones; ◆, magnocellular neurones.

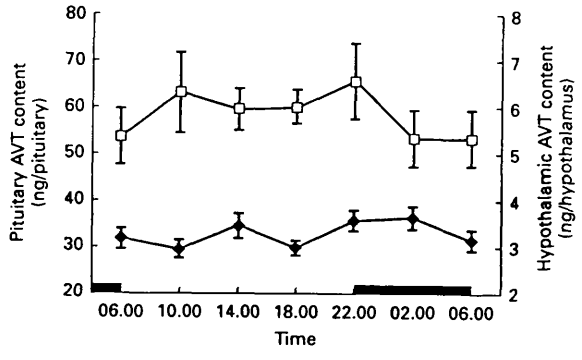

FIG. 3. Hypothalamic and pituitary AVT peptide content over a 24 h cycle. Values (ng/region) are the means (±SEM) of six fish. The 06.00 h value has been repeated for clarity. The thick horizontal bars indicate the dark period. No significant changes were found in either region (ANOVA: $P=0.085$ for the hypothalamus and 0.673 for the pituitary gland). □, pituitary AVT content; ◆, hypothalamic AVT content.

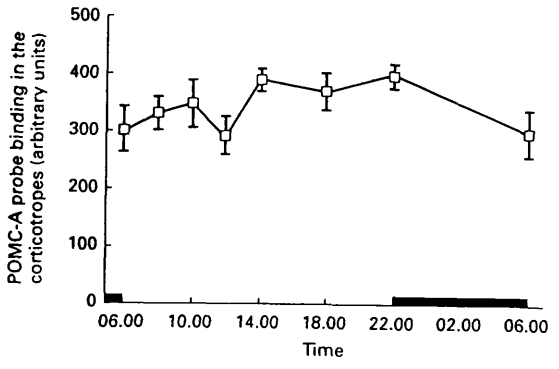

FIG. 4. Binding of POMC-A antisense probe to corticotropes over a 24-h cycle. Values are the means (±SEM) of six fish at each time point. The 06.00 h value has been repeated for clarity. The thick horizontal bars indicate the dark period. No significant changes were evident (ANOVA: $P=0.929$).

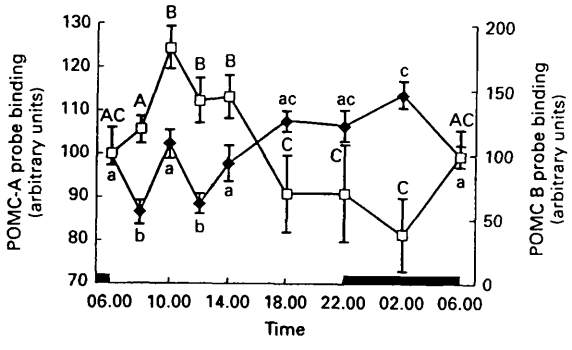

FIG. 5. Binding of POMC-A and POMC-B antisense probes to pars intermedia melanotropes over a 24-h cycle. The normalized values are the means (±SEM) from six to 12 fish at each time point. The 06.00 h value has been repeated for clarity. The thick horizontal bars indicate the dark period. Points labelled with different superscripts (A–C for POMC-A; a–c for POMC-B) are significantly different from one another. Both POMC-A and POMC-B transcripts showed significant changes in abundance over the 24-h period (ANOVA: $P<0.005$). □, POMC-A probe binding; ◆, POMC-B probe binding.

are in complete accord with earlier studies. Previous investigations on trout have shown that AVT and isotocin are produced in both parvo- and magno-cellular neurones of the PON, and that the distribution of the cells producing these two hormones is almost identical (7, 8). More recently Olivereau *et al.* (9–11) have shown, for several teleost species including trout, that the PON also contains irCRF-41 neurones, and this has been confirmed by other studies using immunostaining and *in-situ* hybridization (12). In the eel, and probably in other species, AVT and CRF were shown to co-exist in some neurones and this co-existence became more prevalent following chemical adrenalectomy with metyropone (9, 11). Fibres from these neurones are directed towards the pituitary in which irAVT and irCRF tracts are found near the corticotropes of the pars distalis and in the neural lobe near the melanotropes (11). The PON of fish is therefore the homologue of the paraventricular nucleus (PVN) of mammals albeit that, in teleost fish, fibres project directly into the pituitary rather than terminating on portal vessels.

The marked rise in plasma cortisol during the night implies a coincident secretion of ACTH and one or more of its secretogogues. Theoretically, it is not necessary to assume that all the secretogogues are released at this time, although this could turn out to be the case (particularly, of course, if they are produced in the same cells). Since CRF and AVT act synergistically on trout corticotropes (3), a small increase

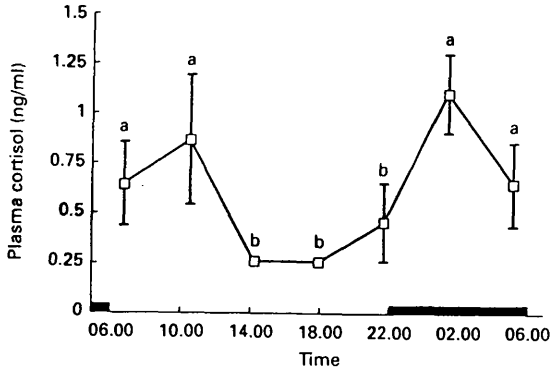

FIG. 6. Diurnal changes in plasma cortisol concentration. Values are the mean (±SEM) of 6–12 fish. Points bearing a different superscript (a,b) are significantly different from one another (χ^2 test, $P < 0.05$).

in the release of just one or other neuropeptide could significantly enhance ACTH release.

The diurnal changes in parvocellular AVT mRNA suggests that the peptide is also released in a cyclical manner and could be involved in the diurnal activity of the hypothalamo-pituitary interrenal axis. The pattern of AVT transcript abundance is almost the inverse of the plasma cortisol concentration. Thus, under the long photoperiod and feeding regime to which these fish were adapted, plasma cortisol was highest at night and early morning and was largely undetectable in the afternoon and evening. The AVT transcripts, conversely, increased progressively during the morning, were highest in the afternoon at the time when cortisol was lowest, and then declined again during the dark period when plasma cortisol was rising. Taken together, the observations would support the interpretation that AVT is released predominantly during the night when it stimulates ACTH secretion, and is replaced by *de novo* synthesis during the day. A similar inverse relationship between CRF mRNA and corticosteroids has been described in rats by some workers (5, 6) although other workers find a slightly different CRF mRNA pattern in which the transcripts increase progressively during the day and are highest at the time of the diurnal corticosterone surge, after which they decline (13).

The inverse relationship between AVT mRNA and cortisol in trout might reflect a negative feedback of the steroid on AVT gene transcription. The possibility of a similar interaction between corticosteroids and CRF mRNA, has been investigated in the rat in which, as mentioned above, CRF mRNA declines either when corticosteroids are rising during the afternoon (5, 6) or immediately after the peak of corticosteroid secretion (13). The results show that a modified diurnal rhythm of CRF mRNA persists in adrenalectomized rats, but that the administration of low levels of corticosterone replacement exert a restraining influence on CRF transcription early on in the photoperiod but is not responsible for the late afternoon decline (13). Thus, in the presence of steroid, the rise in CRF mRNA from its 06.00 h nadir until 16.00 h was gradual after steroid replacement, as in intact rats, whereas in adrenalectomized rats it was steep and rapid,

reaching a peak at 09.00 h. The authors conclude that 'the shape of the diurnal CRF mRNA rhythm in the hypothalamus is largely independent of steroid regulation, although a steroid-dependent component appears to exist during the circadian nadir'. The susceptibility of trout AVT to cortisol feedback remains to be investigated.

Hypothalamic and pituitary AVT peptide showed no significant change in concentration over the 24-h period. It is possible that the amount of AVT released in connection with HPI activity may be a relatively small percentage of the total stored and changes are undetectable for this reason. On the other hand, other workers have shown that circulating AVT also exhibits a diurnal rhythm, hormone levels reaching a peak at the end of the day in trout kept on a short photoperiod (8 L : 16 D) and declining progressively during the night (14). Evening values were up to fivefold higher than in the morning and this evidence of intense peptide release would lead one to anticipate a significant decline in the pituitary AVT content. It seems possible that a similar pattern of hormonal AVT secretion occurs in trout maintained on a long photoperiod, such as ours, although it is uncertain when the peak of plasma AVT would occur—at the end of the day or 8 h after lights on, i.e. 14.00 h. Nevertheless, if the parvocellular AVT neurones not only project to the corticotropes in the pars distalis but also contribute, together with the magnocellular neurones, to hormonal AVT in the circulation, then increased peptide synthesis in these parvocellular neurones during the afternoon could compensate for simultaneous hormonal AVT release, helping to explain the relatively constant abundance of the peptide in the pituitary. Kulczykowska *et al.* (14) found no variation in circulating levels of isotocin throughout the day and it may be relevant in this context that we found no significant change in isotocin mRNA abundance in the parvocellular neurones.

Trout possess two genes for POMC: POMC-A and POMC-B. For these genes, in contrast to the paired AVT and isotocin genes, we have employed selective oligonucleotide probes to monitor their transcription independently. This has previously been done by Salbert *et al.* (15) who showed that the two genes can be regulated differently. Studying the hypothalamic POMC neurones, they found only POMC-A transcripts in immature trout but transcripts of both genes were abundant in sexually mature females or in immature trout following testosterone administration. We now show that the two genes are also differentially regulated in the pituitary, where POMC-A again predominates. Thus both POMC genes showed a diurnal pattern of transcription in the pars intermedia but POMC-A mRNA peaked mid-morning and had its nadir in the middle of the night, while the pattern for POMC-B mRNA was the inverse. Since these were immature fish, sex steroids are unlikely to be involved in these differences but the genes may be differentially responsive to corticosteroids or to some hypothalamic factor. Alternatively, if there is a diurnal pattern of α-MSH release in response to cycles of darkness and illumination, then the transcription changes could be related to this. Given the apparent low abundance of POMC-B mRNA, it is uncertain whether its nocturnal expression has any significance; its translation products are likely to be swamped by those of POMC-A at any time of the cycle. The two genes do not

encode for identical peptides, but it is POMC-A which has the potential to give rise to novel peptides not found in pre-pro-POMC-B (15–17).

There was no evidence of diurnal changes in the level of POMC mRNA transcripts in the corticotropes, in which only POMC-A was detected. If POMC-B is expressed, as reported by other workers (18), it must be at a low level, undetectable in 8 weeks' autoradiographs, whereas POMC-A gave a clear signal in corticotropes after 1 week. Circadian changes in the abundance of mature POMC mRNA have not been observed by all workers studying the rat (19). Those studies which were successful in detecting changes in POMC mRNA abundance employed young rats and demonstrated that such changes become blunted in middle-aged animals (5). Interestingly, the peak of POMC mRNA in rat corticotropes was not in phase with CRH mRNA expression, nor with ACTH release. Such studies show the difficulty of predicting the diurnal pattern of hormone and gene expression in different components of the same physiological axis, even though they are functionally linked.

Materials and methods

Fish

Rainbow trout (*Oncorhynchus mykiss*) were reared from eggs in our aquarium in neutral-coloured tanks, fed by flowing spring water for fry and flowing tap water for older fish. Temperature was 11 °C and photoperiod was 16 L : 8 D with lights on at 06.00 h. Fish were fed daily between 09.00 and 09.30 h. Experimental fish were aged 12 months or 16 months in two separate studies and weighed, respectively, 184 ± 3.7 g or 283 ± 7.3 g (± SEM) at autopsy.

Diurnal rhythms were examined on two occasions, sampling fish at 4 hourly intervals (experiment 1) or at 2 and 4 hourly intervals (experiment 2) throughout the 24-h cycle. Six fish were taken from separate tanks for each time interval and no tank was disturbed more than once. In experiment 1, six additional fish/time intervals were taken for radioimmunoassay of brain and pituitary AVT content.

Collection and preparation of tissues

Fish were deeply anaesthetized in water containing 0.06% phenoxyethanol (Sigma Chemical Co., Poole, Dorset, UK) and 2–3 ml blood from the severed caudal vessels was collected into tubes containing 50 µl 6% Na_2 EDTA as anticoagulant. After decapitation, the brain was exposed, fixative (4% paraformaldehyde in 0.05 M phosphate-buffered saline, pH 7.6) was injected into the ventricles through the optic tectum, and the brain and pituitary were then removed and immersed in the same fixative for 18–24 h at 4 °C. After fixation, the tissue was washed in several changes of distilled water, dehydrated through graded alcohols, cleared in xylene and embedded in wax with plasticizer (Lambwax W/1; Raymond A. Lamb, London, UK). Serial transverse sections of 7 µm (hypothalamus) or 10 µm (pituitary) were mounted in pairs on triplicate sets of gelatinized slides for *in-situ* hybridization.

In-situ hybridization

Hybridization probes

The antisense oligodeoxynucleotide probes for AVT and isotocin have previously been described by Hyodo and Urano (20) for the chum salmon and validated by them for rainbow trout transcripts. Each 46 mer probe corresponded to the mRNA loci encoding proAVT (amino acids −5 to +11) and proisotocin (amino acids −5 to 11). Hyodo and Urano (20) have previously demonstrated these probes bind specifically to their respective RNAs but they would not distinguish between AVT-I and AVT-II, or IT-I and II. The sequences of these probes (synthesized by Perkin Elmer, Warrington, Cheshire, UK) were:

1. AVT: 5′ tt ccc gcc tcg cgg aca gtt ctg gat gta gca cgc aga gga gag cg 3′
2. Isotocin: 5′ tt gcc tcc gat ggg gca gtt gga gat gta gca ggc tgt gca gac ag 3′

The oligoprobes for POMC were based on the cDNA sequences described by Salbert *et al.* (15). Two probes were synthesized that discriminated between the two POMC genes, each 25 mer and complementary to regions in the untranslated 3′ sequences that differ in POMC-A and POMC-B:

1. POMC-A: 5′ ggc tct aag tcc tac agt gac att c-3′
2. POMC-B: 5′ agt ttt caa cag ttg cca ctg gtc t-3′

All probes were labelled at the 3′ end with [α-^{35}S]dATP and purified by QIAquick nucleotide removal kit (Qiagen Ltd, West Sussex, UK).

Procedure

Sections were rehydrated, equilibrated in 2 × SSC at 60 C for 15 min, and then transferred to distilled, de-ionized, filtered water at room temperature. For the hybridization reaction, 100 µl of hybridization mixture (21) containing ^{35}S-labelled oligoprobe (see below) was added to each slide, which was then coverslipped with a rectangle of Nescofilm (BDH, Lutterworth, Leicestershire, UK) and incubated at 37 °C overnight. The composition of the buffer was 50% formamide, 4 × SSC (pH 7.2) containing 500 µg/ml denatured calf thymus DNA, 250 µg/ml yeast tRNA, 1 × Denhardt's solution (0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 10% dextran sulphate (MW 500 000), and 500 mM dithiothreitol (all from Sigma Chemical Co. Ltd, St Louis, MO, USA). After hybridization, sections were given four rinses in 1 × SSC at room temperature, followed by four washes for 15 min each in 1 × SSC at 60′ C and 2 washes for 30 min each in 1 × SSC at room temperature. Following a rinse in distilled water, slides were dried at 37 °C and then apposed to autoradiographic film (Hyperfilm MP, Amersham International plc, Buckinghamshire, UK). Films were exposed for 3–9 days, depending on the probe and cell type.

Analysis of in-situ hybridization

The density of autoradiographic signal was determined by computerized densitometry (21). A standard curve, constructed from the autoradiographic signal of ^{14}C standard strips (microscales, Amersham, UK) allowed the non-linear response of the film to irradiation to be taken into account.

For the parvocellular AVT neurones (parvo-AVT) located in the PON, preliminary trials showed that a good representation of the whole was obtained by scanning the autoradiographic signal from six sections/brain, selected half way along the anterior-posterior mass of parvo-AVT neurones. This region does not include any magnocellular neurones, dorsally, nor the more anterior periventricular AVT neurones of the PON above the preoptic recess. The mean values of 6–12 brains per time point were used to determine the relative abundance of parvo-AVT gene transcripts. To assess magnocellular AVT neuronal activity, all perikarya located along the longitudinal axis, posterior to the main parvocellular mass (Fig. 1) were scanned. To monitor POMC mRNA in the pituitary, the signals from corticotropes in the pars distalis and melanotropes in the neurointermediate lobe were scanned in 12 median sagittal sections/pituitary gland.

Cortisol radioimmunoassay

Cortisol was measured using a slight modification of a procedure described previously (1). Briefly, 100 or 200 µl plasma from each fish was extracted into 1 ml ethanol, and duplicate 400 µl aliquots of extract were placed in polypropylene tubes and dried down under vacuum. A mixture of ^{3}H-cortisol (Amersham plc; 20 000 d.p.m./assay tube) and cortisol antiserum (Bath-R5, diluted 1 : 4000) was added to each assay tube in 200 µl of phosphate buffer pH 7.6 containing 0.8% NaCl and 0.1% gelatin. After overnight incubation, bound and unbound cortisol were separated using dextran-coated charcoal. The supernatant (antibody-bound cortisol) was tipped into Optiphase Safe scintiliant (Wallac Scintillation products, Leicestershire, UK) and counted in a scintillation counter. The antiserum was relatively specific for cortisol (cortisol 100%; corticosterone 2.09%; cortisone 0.26%; progesterone 0.009%; testosterone 0.008%)

Arginine vasotocin radioimmunoassay

Pituitaries and hypothalami were sonicated in 500 µl of 0.1 M HCl. The extract was centrifuged and the supernatant stored at −20 °C until assay. Arginine vasotocin peptide (Sigma) was used as a standard. Following iodination by the chloramine T method, the labelled peptide was applied to a Sep-pak (Waters Associated, Milford, MA, USA), and eluted using graded concentrations of acetonitrile containing 0.1% TFA (trifluoroacetic acid, Sigma). The eluant containing ^{125}I-AVT was diluted 10-fold in assay buffer (Phosphate-buffered saline with 1% BSA) and stored in 50 µl aliquots at −20 °C. The antiserum (kindly donated by Dr P. Ingleton) was used at a final dilution of 1 : 13 333. Bound and unbound AVT were separated using

dextran-coated charcoal. The assay detected between 15 and 1000 pg AVT/tube. The antiserum showed cross-reactivity with isotocin at 0.014% compared with AVT (100%).

Immunocytochemistry

Trout brains were fixed in sublimated Bouin-Holland fixative for 6 days and then embedded in wax as described above. Sections were cut into 5-μm sections and immunostained for AVT using a VectaStain Elite ABC kit (Vector Laboratories, California, USA). The AVT antiserum was used at a 1:1000 dilution. The peroxidase was visualized using diaminobenzidene as substrate. Corticotropin-releasing factor was similarly located with a specific CRF antiserum, generously provided by Professor P. Lowry, used at a 500-fold dilution.

Statistical analysis

Log-transformed data were analysed using one way ANOVA. When this indicated a statistical difference ($P<0.05$), it was followed by Fisher's LSD test. A χ^2 test was used to inspect for diurnal differences in plasma cortisol concentration.

Acknowledgements

The authors thank Dr P. Ingleton and Professor P. Lowry for their generous provision of antisera. This work was supported by a grant from BBSRC.

Accepted 22 June 1998

References

1 Baker BI, Rance TA. Differences in concentration of plasma cortisol in the trout and the eel following adaptation to black or white backgrounds. *J Endocr* 1981; **89**: 135–140.

2 Pickering A, Pottinger T. Seasonal and diel changes in plasma cortisol levels of the brown trout, *Salmo trutta* L. *Gen Comp Endocrinol* 1983; **49**: 232–239.

3 Baker BI, Bird DJ, Buckingham JC. In the trout, CRH and AVT synergize to stimulate ACTH release. *Reg Peptides* 1996; **67**: 207–210.

4 Fryer J, Lederis K, Rivier J. ACTH-releasing activity of urotensin I and ovine CRF: interactions with arginine vasotocin, isotocin and arginine vasopressin. *Reg Peptides* 1985; **11**: 11–15.

5 Cai A, Scarbrough K, Hinkle D, Wise P. Fetal grafts containing chiasmatic nuclei restore the diurnal rhythm of CRH and POMC mRNA in aging rats. *Am J Physiol* 1997; **273**: R1764–R1770.

6 Watts A, Swanson L. Diurnal variation in the content of preprocorticotropin-releasing hormone messenger ribonucleic acids in the hypothalamic paraventricular nucleus of rats of both sexes as measured by *in-situ* hybridization. *Endocrinology* 1989; **125**: 1734–1738.

7 Goossens N, Dierickx K, Vandesande F. Immunocytochemical localization of vasotocin and isotocin in the preopticohypophysial neurosecretory system of teleosts. *Gen Comp Endocrinol* 1977; **32**: 371–375.

8 Van den Dungen H, Buijs R, Pool C, Terlou M. The distribution of vasotocin and isotocin in the brain of the rainbow trout. *J Comp Neurol* 1982; **212**: 146–157.

9 Olivereau M, Moons L, Olivereau J, Vandersande F. Coexistence of corticotropin-releasing factor-like immunoreactivity and vasotocin in perikarya of the preoptic nucleus in the eel. *Gen Comp Endocrinol* 1988; **70**: 41–48.

10 Olivereau M, Olivereau J. Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. *Peptides* 1988; **9**: 13–21.

11 Olivereau M, Olivereau J. Effect of pharmacological adrenalectomy on corticotropin-releasing factor-like and arginine vasotocin immunoreactivities in the brain and pituitary of the eel: immunocytochemical study. *Gen Comp Endocrinol* 1990; **80**: 199–215.

12 Okawara Y, Ko D, Morley SD, Richter D, Lederis KP. *In-situ* hybridization of corticotropin-releasing factor-encoding messenger RNA in the hypothalamus of the white sucker, *Catastomus commersoni*. *Cell Tiss Res* 1992; **267**: 545–549.

13 Kwak S, Morano M, Young E, Watson S, Akil H. Diurnal CRHmRNA rhythm in the hypothalamus: decreased expression in the evening is not dependent on endogenous glucocorticoids. *Neuroendocrinology* 1993; **57**: 96–105.

14 Kulczykowska E, Stolarski J. Diurnal changes in plasma arginine vasotocin and isotocin in rainbow trout adapted to freshwater and brackish Baltic water. *Gen Comp Endocrinol* 1996; **104**: 197–202.

15 Salbert G, Chauveau I, Bonnec G, Valotaire Y, Jego P. One of the two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol Endocrinol* 1992; **6**: 1605–1613.

16 Tollemer H, Leprince J, Bailhache T, Chauveau I, Vandesande F, Tonon MC, Jego P, Vaudry H. Characterization of a novel alpha-amidated decapeptide derived from proopiomelanocortin-A in the trout pituitary. *Endocrinology* 1997; **138**: 128–137.

17 Tollemer H, Leprince J, Galas L, Vandesande F, LeMevel JC, Tonon MC, Conlon JM, Vaudry H. Isolation and structural characterization of two novel peptides derived from proopiomelanocortin in the pituitary of the rainbow trout. *Biochem Biophys Res Comm* 1997; **238**: 653–657.

18 Chauveau I, Salbert G, Bunel DT, Vaudry H, Valotaire Y, Jego P. Cloning and sequencing of two POMC cDNAs in rainbow trout (*Oncorhynchus mykiss*): effect of sex steroids on the expression of the POMC genes. *Proc Natl Acad Sci USA* 1993; **680**: 476–477.

19 Kwak S, Young A, Akil H, Watson S. Changes in proopiomelanocortin primary transcript levels in the anterior pituitary accompany increased adrenocorticotropin secretion during diurnal surge. *J Neuroendocrinol* 1992; **4**: 21–27.

20 Hyodo S, Urano A. Changes in expression of provasotocin and proisotocin genes during adaptation to hyper- and hypo-osmotic environments in rainbow trout. *J Comp Physiol B* 1991; **161**: 549–556.

21 Suzuki M, Narnaware YK, Baker BI, Levy A. Influence of environmental colour and diurnal phase on MCH gene expression in the trout. *J Neuroendocrinol* 1995; **7**: 319–328.

3.3 Additional Discussion

Many of the issues raised by this study have already been discussed in the published paper. However, a number of topics require amplification in light of more recent publications and further interpretation. To put the additional discussion into context a brief summary is given here of the main points already covered. The pattern of AVT transcript abundance in the parvocellular preoptic nucleus shows a diurnal rhythm, with values being highest during the light period. This is almost the inverse of that of plasma cortisol concentration, which peaks during the night. It seems that either AVT transcription during the day is compensating for peptide released during the dark period when it stimulates ACTH secretion, or that cortisol is having a negative feedback effect on AVT mRNA levels at night. Though AVT peptide concentration in the hypothalamus and pituitary show no significant variation over the 24 hour period, the changes may be too small to be detected if the amount released represents a small percentage of the total stored. In addition, if the parvocellular AVT perikarya project, along with the magnocellular neurones, to the neurointermediate lobe (NIL) then increased peptide synthesis in the parvocellular AVT perikarya during the afternoon could compensate for the simultaneous release of AVT into the circulation (Kulczykowska & Stolarski, 1996), and explain the relatively constant abundance of peptide in the pituitary. In the pars intermedia the POMC genes, A and B, display inverse patterns of transcription suggesting differential regulation; this may be due to variations in their responsiveness to corticosteroid, a result of the influence of some hypothalamic factor, or a differential contribution to the diurnal pattern of α-MSH release. There was no diurnal change in POMC-A in the corticotropes, though in rats such a rhythm is not always present either (Kwak *et al.*, 1992).

3.3.1 Tetraploidy and differential regulation of gene transcripts

Salmonid fish are tetraploid and consequently have duplicate genes for all hypothalamic and pituitary hormones. In some cases one form of the gene is expressed predominantly or exclusively. This seems to be true of arginine vasotocin (AVT), isotocin (IT), and melanin concentrating hormone (MCH). Although both AVT-1 and AVT-2 gene

transcripts are detectable in rainbow trout the expression of the latter is scarce (Hiraoka *et al.*, 1993). Incidence of IT-1 tends to be greater than that of IT-2 in most species of the *Oncorhynchus* genus, including rainbow trout (Suzuki *et al.*, 1992; Hiraoka *et al.*, 1993). With respect to MCH-1 and MCH-2, probes specific to the former in rainbow trout have failed to detect its presence (Baker *et al.*, 1995; Francis *et al.*, 1997), though the latter is clearly visible. This is also the case in the coho salmon, *O.kisutch* (Nahon *et al.*, 1991). However, in the chum and chinook salmon, *O.keta* (Ono *et al.*, 1988) and *O.tshawytcha* (Minth *et al.*, 1989), both are expressed. Although it cannot be discounted that either form of AVT, IT, or MCH gene transcripts may respond independently under certain conditions, there is no evidence to suggest this is the case. Consequently probes that did not distinguish between the respective gene duplicates were used. However, the POMC genes, A and B, have been found to be differentially regulated by sex steroids in the trout when expressed in hypothalamic neurones (Chauveau *et al.*, 1993). POMC-A is expressed by both mature and immature animals whilst POMC-B is detectable only in the hypothalami of sexually active individuals. Given this evidence probes specific to both forms of the gene were used to study the pituitary.

In the current work we found a variation in the expression of POMC-A and POMC-B in the pituitary pars intermedia. As in the hypothalamus (Salbert *et al.*, 1992) POMC-A is the dominant form. Though both exhibited a diurnal variation we found that POMC-A and B displayed inverse patterns of expression. POMC-A transcripts were highest in the morning, declining to a night-time nadir, whilst POMC-B mRNA progressively increased through the day and peaked during the dark period. It is possible that this reflects differences between the two genes in sensitivity to corticosteroid negative feedback, with POMC-B the more susceptible of the two genes. The diurnal variation in melanin-concentrating hormone (MCH), which peaks at midday, may also play a role as in the trout it inhibits the release of melanocyte-stimulating hormone, α-MSH (Baker *et al.*, 1986), one of the cleavage products of the POMC gene in the pars intermedia (Barber *et al.*, 1987). In rainbow trout MCH is involved in physiological colour change and is one of the functional antagonists of α-MSH (Baker, 1988). MCH exhibits a clear circadian rhythm with maximal levels at midday, before declining to low levels during the night (Lyon & Baker, 1993). This is reflected in the hypothalamic MCH gene transcripts which are high at 16.00h and low at 04.00h (Suzuki *et al.*, 1995). In contrast, circulating

α-MSH is low in the middle of the day in fish kept in pale-coloured tanks, with higher levels at night (Baker; personal communication). Thus, on a white background, such as that which fish in this study were kept, plasma concentration was 266±38pg/ml. Fish kept in black tanks had higher levels (560±70pg/ml) as one would expect. Values during the dark period increased to 589±76pg/ml, which is likely to be attributable to a diurnal surge. Whatever the control mechanisms, the evidence suggests the two POMC genes are differentially controlled, and also exhibit a diurnal rhythm of expression.

In the corticotropes no diurnal variation in POMC transcripts was evident. Indeed, despite extended autoradiograph exposure POMC-B was not even detected. The reasons for this are unclear since other workers have found POMC-B is expressed in the corticotropes (Chauveau *et al.*, 1993). Despite the evidence of differential regulation of the two POMC genes in melanotropes future work focused on stress and consequently corticotrope responses. Thus, a probe that did not distinguish between the two forms of the POMC gene was used in further experiments to ensure the contribution of POMC-B to the stress response was included in analysis, albeit probably at very low levels.

3.3.2 The role of cortisol negative feedback in the control of AVT transcripts

Interestingly the pattern of AVT transcript abundance was almost the inverse of that of plasma cortisol concentration. Plasma cortisol concentration decreases into the afternoon, as parvocellular transcripts rise, before increasing again through the dark period as AVT mRNA declines once more. This could be a result of corticosteroid negative feedback. In rats, though the shape of the diurnal CRH mRNA rhythm in the hypothalamus is largely independent of corticosteroid regulation, a steroid-dependant component appears to exist during the morning circadian nadir (Kwak *et al.*, 1993). Immunocytochemical studies on the eel indicate an increase in both irAVT and irCRH abundance and their co-expression following the removal of cortisol by chemical or surgical interrenalectomy (Olivereau & Olivereau, 1990*b*, 1991*a*) or hypophysectomy (Olivereau & Olivereau, 1988*b*) suggesting cortisol has a restraining influence on the synthesis of these peptides in fish. Moreover, it has been recently shown that there are numerous glucocorticoid receptors in the rainbow trout brain (Teitsma *et al.*, 1997, 1998), with all irCRH neurones, many of which also contain AVT, expressing such receptors.

How, or indeed if, cortisol acts on AVT transcription in the trout could only be determined by monitoring AVT transcripts after chemical adrenalectomy. This is discussed further in Chapter 5.

3.3.3 The parvocellular AVT mRNA rhythm; circulating peptide or corticotrope control?

Although cortisol negative feedback might play a role in the diurnal control of AVT transcription, the parvocellular mRNA rhythm may be more related to diurnal release of AVT into the circulation. Immunoreactive fibres for both AVT and CRH are found in the neurointermediate lobe (NIL) (white sucker, *Catastomus commerson*: Yulis & Lederis, 1987; *Anguilla sp.*: Olivereau *et al.*, 1988; rainbow trout: Olivereau & Olivereau, 1990*a*; chinook salmon, *Oncorhynchus tshawytsch*: Matz & Hofeldt, 1999) though their origin is not clear. It seems probable most of these arise from the magnocellular AVT, CRH, and IT neurones since their large size suggests they are functionally designed for peptide release into the plasma. However, the parvocellular AVT perikarya may also project to the NIL and contribute to circulating peptide. The amount of peptide required to regulate corticotrope activity is probably very small yet the number of parvocellular AVT neurones, and the abundance of AVT transcripts in this area, seems disproportionate to this single role. In terms of peptide the total quantity of AVT, from immunocytochemical studies, appears to exceed that of the magnocellular perikarya. Total AVT transcript abundance is, indeed, greater in parvo- than magno- cellular neurones. Furthermore, the diurnal variation of AVT transcripts in the parvocellular neurones overlaps with that of the circulating peptide which rises through the day and peaks at the start of the dark period before declining again (Kulczykowska & Stolarski, 1996). Transcripts rise to reach a peak at midday, remaining high until decline during the night. Thus, AVT gene expression may be more closely associated with the release and replenishment of posterior pituitary lobe AVT than with cortisol secretion. If this is the case, then the parvocellular region consists of a heterogeneous population of perikarya with respect to where the axons terminate. Those that project to the NIL and contribute, with the magnocellular neurones, to circulating AVT which influences blood pressure (Le Mevel *et al.*, 1993; Warne & Balment, 1997), and those which project to the pars distalis and are part of the HPI axis. Both of these, of course, may respond to stress; this is discussed in further detail in Chapter 4. This is not to say that the AVT mRNA rhythm observed in

the parvocellular perikarya does not contain a component involved in corticotrope control. Since plasma cortisol levels are extremely low (for the most part on the limits of detection), which would require very little ACTH release in order to stimulate, the contribution of corticotrope-controlling AVT transcripts to the total parvocellular AVT mRNA pool may be very small. In this case, this rhythm may be masked by that of those parvocellular AVT neurones that contribute towards circulating peptide. Nevertheless, since it is impossible to differentiate between the two types of parvocellular neurones, future stress experiments monitoring parvocellular AVT mRNA treated the nucleus as a single, functional unit and measured transcripts only during their diurnal plateau.

3.3.4 Hypothalamic and pituitary AVT peptide content

Despite a reported fivefold difference in circulating AVT concentration between the early morning nadir and night time peak (Kulczykowska & Stolarski, 1996), which one would expect to be reflected in the amount of AVT peptide in both the pituitary and hypothalamus, there were no such changes over the 24 hour period in the current study. It may be that any variations are difficult to detect as released product may comprise a small percentage of the amount stored. Alternatively the variations in AVT content between fish may negate any possible significant differences. Furthermore, if parvocellular AVT neurones contribute along with the magnocellular neurones to circulating peptide, as speculated in Section 3.3.3, then this may explain the lack of any discernible rhythm in pituitary content as peptide released would be compensated for by the increased levels of AVT synthesis in parvocellular neurones in the afternoon. Clear changes in hypothalamic AVT content have been reported in goldfish with significantly higher concentrations at 22.00h than 10.00h (Hontela & Lederis, 1985). This matches differences found in both transcripts of the present study and that of Kulczyowska and Stolarkis' (1996) plasma peptide work.

3.3.5 The lack of diurnal changes in isotocin transcripts

Parvocellular isotocin mRNA showed no significant change in abundance over the 24 hour period. This is could be related to the fact that circulating levels of isotocin do not display a diurnal rhythm (Kulczykowska & Stolarski, 1996). Isotocin's role is not clearly understood at present and has been little studied. It may play only a minor role in ACTH

secretion, though it has been implicated in salt and water metabolism (Pierson *et al.*, 1996) as well as the control of stress (Fryer *et al.*, 1985). The involvement of isotocin in the latter of these is investigated in further detail in Chapter 4.

3.3.6 Summary

In summary it seems that considerable difficulty lies in the coupling of diurnal transcription rates of functionally linked peptides. The determination of the CRH mRNA rhythm might serve to clarify the relationships of the hypothalamo-pituitary-interrenal axis since the product is a more potent ACTH secretogogue. Based on this work future stress-response studies were carried out when both AVT transcripts and plasma cortisol concentration plateau (i.e. between 10.00 and 22.00h).

Chapter 4

Gene Transcript Responses to Acute and Chronic Stress

4.1 Introduction

In fish, a CRH has been identified which shares strong evolutionary and functional links with its mammalian counterpart (Lovejoy & Balment, 1999). As in rats, CRH and AVT are the major known ACTH secretogogues in the trout (Baker *et al.*, 1996). Both parvo- and magno- cellular CRH and AVT positive neurones have been identified in the pre-optic nucleus (PON) of the trout (Van den Dungen *et al.*, 1982; Olivereau & Olivereau, 1988*a*, 1990*a*) which is generally accepted as the homologue of the mammalian stress centre, the paraventricular nucleus (Fryer & Peter, 1975). Fish lack a conventional hypophysial portal system, thus fibres from these PON neurones extend posteriorly and terminate in the pituitary, providing some insight into which cell types CRH and AVT influence. Since immunoreactive fibres of both these neuropeptides have been found near the pituitary corticotropes and melanotropes (Yulis & Lederis, 1987; Van den Dungen *et al.*, 1982; Olivereau & Olivereau, 1988*a*, 1990*a*; Matz & Hofeldt, 1999) there is strong evidence to suggest they may act on these pituitary cell types. Taken together it appears that CRH and AVT may play a central role in the stress response in fish.

In rats, CRH is generally accepted as the primary ACTH secretogogue following a single stress (Lightman & Harbuz, 1993; Makino *et al.*, 1995), whilst AVP plays a more central role when such stressors are applied chronically (i.e. repeatedly). The neuropeptide response to acute stress is thus not necessarily mimicked by the response to chronic stress. In the rat there generally is a transcript-specific alteration in gene regulation whereby CRH habituates and AVP transcription is maintained (Ma *et al.*, 1997*a*), presumably to maintain sensitivity of the HPA to further stressful events. Such studies on either acute or chronic stress have yet to be carried out in fish.

Whether isotocin has a role in the stress response in fish is not currently known though there is some evidence to suggest that it is involved. It has been shown to stimulate ACTH release from cultured trout pituitary cells, albeit weakly (Pierson *et al.*, 1996), with immunoreactives located in an almost identical distribution throughout the PON to that of AVT (Van den Dungen *et al.*, 1982).

Although ACTH is the primary pituitary corticotrope hormone responsible for stimulating cortisol release from the interrenal, there is some evidence to suggest that α-MSH, the principal hormone released from the pituitary melanotropes, may also have a function with respect to the control of cortisol release. Thus, under certain conditions of stress, the corticotropes do not respond though the melanotropes are stimulated, with a concurrent rise in plasma α-MSH and cortisol (Arends *et al.*, 1999). Whether α-MSH is influencing cortisol release in the absence of ACTH in such instances is unclear.

Aims

The work described within this chapter investigates the response of AVT and POMC, and to a certain extent CRH and IT, transcripts to stress. Initially (published paper) a number of stressors were applied both acutely and chronically to observe transcript responses under such conditions. This also served to determine if there is an alteration in mRNA regulation when a stress is applied repeatedly as opposed to singly, such as occurs in the rat. Additional experiments sought to gain a better understanding of these transcripts' responses to acute stressors of different duration and magnitude of intensity.

Finally, a number of sequence probes, techniques and conditions were tested in an attempt to rationalise a suitable *in situ* hybridisation technique for CRH in our fish. This was based on recent work in which CRH mRNA had been successfully detected in rainbow trout using a sequence complimentary to sockeye salmon CRH (Ando *et al.*, 1999). Since duplicate tissue samples were taken for all experiments carried out previous to the discovery of the CRH sequence in salmon it was hoped that this method could be used to determine CRH mRNA levels in all prior experiments. Since CRH is a more potent ACTH-secretogogue than AVT (Baker *et al.*, 1996) this would serve to improve our understanding of the stress response in the trout.

4.2 Published Paper

The Effects of Acute and Chronic Stresses on Vasotocin Gene Transcripts in the Brain of the Rainbow Trout (*Oncorhynchus mykiss*)

Gilchriest, B.J., Tipping, D.R., Hake, L., Levy, A., Baker, B.I.
Journal of Neuroendocrinology, 2000, Vol. 12, 795-801.

In this paper the majority of the work described was carried out by B.Gilchriest. *In situ* hybridisations for POMC were carried out by Dr. D.Tipping. L.Hake assited as a laboratory technician on this work. Facilities for labelling oligonucleotide probes were provided for by A.Levy. Dr. B.I.Baker supervised the work.

Journal of Neuroendocrinology, 2000, Vol. 12, 795–801

The Effects of Acute and Chronic Stresses on Vasotocin Gene Transcripts in the Brain of the Rainbow Trout (*Oncorhynchus mykiss*)

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Key words: *Oncorhynchus mykiss*, acute and chronic stress, *in situ* hybridization, arginine vasotocin mRNA, POMC mRNA, cortisol secretion.

Abstract

Secretion of adrenocorticotropic hormone (ACTH) from the fish pituitary, which occurs in times of stress, is stimulated by several hypothalamic neuropeptides, one of which is arginine vasotocin (AVT). This study investigates whether gene expression for AVT is up-regulated during acute or chronic stress. Rainbow trout (*Oncorhynchus mykiss*) were subjected to one of two forms of acute stress—either 2 h confinement followed by 2 h recovery, or capture and transfer to low water for 2 min followed by 4 h recovery in their home tank before autopsy. In other experiments, these stresses were repeated daily for 5 or 6 days (chronic stress). Quantification of AVT transcript prevalence in the parvocellular and magnocellular neurones of the preoptic nucleus after *in situ* hybridization was used as a monitor of the AVT gene response to stress. The results showed that acute confinement, but apparently not brief low-water stress, significantly increased AVT transcript prevalence in a group of parvocellular perikarya. When applied repeatedly, both forms of stress caused habituation, such that the AVT hybridization signal remained at control or even lower levels despite elevated pro-opiomelanocortin transcripts in the corticotropes and raised plasma cortisol concentrations. The AVT hybridization signal in the magnocellular perikarya showed no significant response to either acute or chronic stress. The results support the idea that these parvocellular AVT neurones are involved in ACTH stimulation during acute stress, and that the system habituates to chronic stresses.

In mammals, the corticosteroid response to stress is initiated by the release of the hypothalamic hormones corticotropin-releasing factor (CRF) and vasopressin into the pituitary portal vessels. These peptides trigger the release of corticotropin (ACTH) from the pituitary and hence increase the secretion of corticosteroids. If the stress is sufficiently severe, this cascade of events is usually accompanied by increased transcription and translation of the stress hormones.

Quantitative *in situ* hybridization has proved a valuable tool for defining which stresses modulate transcription of the various genes involved. Studies show that, although vasopressin is colocalized with CRF in many parvocellular neurones, transcription of the two neuropeptides can be independently controlled. For example, electroconvulsions (1) or immobilization/restraint (2–4) will increase the abundance of both CRF and vasopressin mRNA; only CRF mRNA may increase after acute hypotension (5) or i.c.v. infusion of histamine (6) whereas vasopressin mRNA alone increases during adjuvant-induced arthritis (7) when CRF mRNA declines. Such studies also show that stresses which enhance transcript numbers when applied acutely may no longer have this effect when they are repeated daily (4, 8).

Non-mammalian vertebrates possess a hypothalamo-pituitary-adrenal system very similar to that of mammals. In the case of teleost fish, CRF and homologues of vasopressin—arginine vasotocin (AVT) and isotocin are usually all expressed in the preoptic nucleus of the forebrain (9–15) and will stimulate ACTH release from goldfish or trout pituitary glands (16–18). As in rats, CRF is the more potent peptide but in the trout, AVT can synergize with it to enhance ACTH release (17).

It is not known how stress affects biosynthesis of these neuropeptides in fish. The present study uses quantitative *in situ* hybridization to investigate the effects of stress of either

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2 min or 2 h duration, applied either once or repeatedly, on the abundance of AVT in the preoptic neurones. Plasma cortisol concentration and pituitary pro-opiomelanocortin (POMC) mRNA were also monitored as indicators of stress.

Materials and methods

Fish

Rainbow trout (*Oncorynchus mykiss*) were reared from eyed eggs in our aquarium in pale-coloured tanks at 11 °C, using flowing spring water for young fish and flowing tap water after about 5 months. Light : dark photoperiod was 16 h:8 h with lights on at 06.00 h. Young fish were fed thrice daily, starting at 09.00 h, and this was gradually reduced to once daily at approximately 6 months. At this point, fish were transferred to 450 l tanks with a stocking density of 19–28 g/l.

Experiments

All fish used in any one experiment were always taken from the same 450 l stock tank, where they were left undisturbed for several weeks beforehand. To avoid diurnal fluctuations, all stress experiments were started at approximately 14.00 h since earlier work (19) demonstrated constant AVT and POMC transcript abundance between 14.00 h and 22.00 h. Fish were captured by a sweep of a large net and subjected either to 2 h confinement stress or to 2 min in low water. Confinement stress consisted of transferring fish to white buckets (28 cm diameter) containing 15 l of aerated flowing water. After 2 h confinement, they were transferred to 450 l recovery tanks (filled with water from their home tank) and sacrificed 2 h or more after recovery. Low water stress consisted of netting fish into a tank 45 × 30 cm with water to a depth of approximately 4 cm, such that they were unable to remain upright. After 2 min, they were tipped back into a 450-l recovery tank and sacrificed 4 h later. All fish withstood the stresses satisfactorily.

Experiment 1

Control fish were anaesthetized and blood, brains and pituitaries were processed as described below. At the same time, 24 fish from the same tank were confined in buckets at 56 g/l for 2 h. They were then returned to four separate recovery tanks (6 fish per tank) and sacrificed 2 h, 6 h or 26 h later. Fish in the fourth tank were subjected to a daily 2 h confinement stress between 14.00 h and 16.00 h and were sacrificed after 2 h recovery on the fifth day. Fish weight was 261 ± 7 g.

Experiment 2 (acute stress)

Eight fish (mean weight 403 ± 15 g) were stressed by 2 h confinement in buckets at 214 g/l. Eight others from the same stock tank were stressed by transfer to low water for 2 min as described above. Stressed fish were sacrificed, together with controls, at 18.00 h, 4 h after the start of stress.

Experiment 3 (repeated stress)

This experiment was carried out in parallel with experiment 2. Eight fish (mean weight 398 ± 15 g), taken from the same stock as experiment 2 to avoid tanks effects, were stressed by confinement (212 g/l) or by low water, and returned to recovery tanks. The stress was repeated daily and fish were sacrificed on the sixth day at 18.00 h, i.e. 4 h after the start of stress. Eight control fish were left undisturbed in the original stock tank and sacrificed at the same time as the stressed fish.

Collection and preparation of tissues

Fish were deeply anaesthetized in 0.06% phenoxyethanol (Sigma Chemical Co., Poole, Dorset, UK) and 2–3 ml of blood collected from the severed caudal fin into chilled tubes containing 50 µl of 6% Na_2EDTA as anticoagulant. Following decapitation, the brain was exposed and ice-cold fixative (4% paraformaldehyde in 0.05 M phosphate buffer pH 7.6 containing 0.8% NaCl) was injected into the ventricles through the optic tectum. Brains and pituitaries were removed and postfixed for 18–24 h in the same fixative at 4 °C. After fixation, tissues were washed in several changes of distilled water, dehydrated in graded ethanol, cleared in xylene and embedded in wax containing plasticizer (Lambwax W/1; Raymond A. Lamb, London, UK). Serial 10 µm pairs of sections of the brain (transverse sections) and pituitary (sagittal sections) were mounted on duplicate sets of gelatinized slides.

In situ *hybridization*

The antisense oligodeoxynucleotide probes for AVT, which does not bind to isotocin transcripts, has been described previously (19, 20). The oligoprobe for POMC was complementary to an exonic sequence common to POMC A and POMC B mRNAs (21, 22). All oligoprobes were synthesized by Perkin Elmer (Warrington, Cheshire, UK). They were labelled at the 3′ end with [α-^{35}S]-dATP and purified in phenol–chloroform.

The hybridization procedure was as described previously (19). Briefly, sections were dewaxed and rehydrated prior to overnight incubation at 37 °C with hybridization buffer containing one of the ^{35}S-labelled probes at either 60,000 cpm/100 µl/slide (AVT) or 100 000 cpm/100 µl/slide (POMC). Following hybridization, sections were given four rinses in 1 × SSC at room temperature, four 15 min washes in 1 × SSC at 60 °C. This was followed by two 30 min washes in 1 × SSC and a rinse in distilled water, both at room temperature.

The autoradiographic film (Hyperfilm, Amersham International, plc, Bucks, UK) was exposed to hybridized sections for between 2 and 21 days, depending on the cell type under study. Autoradiographic signals were quantified by computer densitometry, using 'NIH image' on a Mac IIci computer. High and low ^{14}C standards (Microscales, Amersham) were used to construct a standard curve of signal intensity versus radioactivity.

For parvocellular AVT neurones, a good representation of the whole was obtained by scanning the autoradiographic signals from eight sections/brain, located midway along the anterior–posterior range of these neurones. This region avoided the ventral parvocellular neurones over the preoptic recess and also avoided the magnocellular neurones more dorsally. All clusters of magnocellular neurones, located along the anterio-caudal axis, posterior to the main parvocellular mass, were also scanned. To monitor POMC expression in the pituitary, the signals from 12 median sagittal sections/pituitary gland were scanned for both the corticotropes in the pars distalis, and melanotropes in the neurointermediate lobe.

Cortisol radioimmunoassay

The cortisol radioimmunoassay in plasma samples has been described previously (19).

Statistical analysis

Log-transformed data were analysed by ANOVA. If this showed a significant difference ($P < 0.05$), results were further analysed by Fisher's LSD test. In some cases, data were analysed by the non parametric Mann–Whitney U-test.

Results

Effect of different stresses on AVT hybridization signal in parvocellular neurones

Preliminary trials investigated the cortisol response when fish were confined for 2 h in a bucket with flowing water, followed by a 2-h recovery period in their home tank. After a few minutes of initial struggling, fish became completely quiet. Plasma cortisol concentrations were high at the end of the 2 h stress period and then declined significantly during the 2 h recovery period although they had not returned to basal. A typical example of cortisol titres was: (i) unstressed controls, 2.4 ± 1.3 ng/ml; (ii) 2 h stress, 53.5 ± 4.7 ng/ml; (iii) 2 h stress plus 2 h recovery, 11.9 ± 2.3 ng/ml ($n = 8$). Higher confinement densities tended to be associated with higher plasma cortisol concentrations although the regression correlation was not statistically significant (cortisol *vs* g/l; $r^2 = 0.42$; $n = 19$). Several experiments using confinement stress were performed.

The location of AVT neurones under discussion is shown in Fig. 1, which also shows binding of the complementary AVT probe to preoptic neurones, and the POMC probe to corticotropes and melanotropes in the pituitary gland.

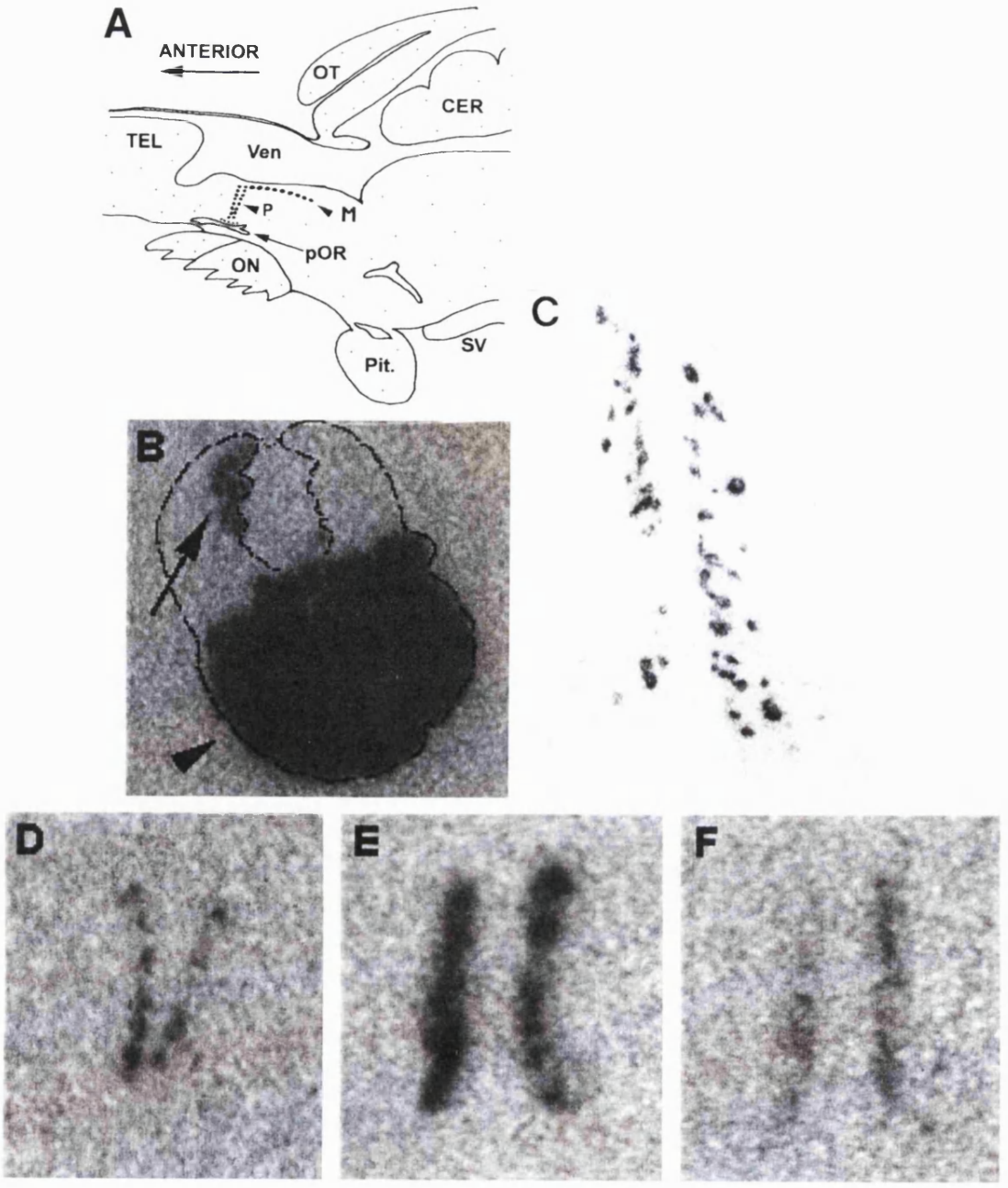


FIG. 1. (A) Diagrammatic parasagittal section of the trout brain showing the location of the different groups of arginine vasotocin (AVT) perikarya in the preoptic nucleus. CER, cerebellum; M, magnocellular neurones; ON, optic nerve; OT, optic tectum; P, parvocellular neurones; Pit, pituitary; pOR, preoptic recess; SV, saccus vasculosus; V, ventricle. (B) X-ray film autoradiograph of a sagittal section through the trout pituitary showing pro-opiomelanocortin hybridization signals in the corticotropes (arrow) of the pars distalis, and in the melanotropes (arrowhead) of the neurointermediate lobe. (C) Emulsion-dipped autoradiograph showing the AVT hybridization signal in parvocellular neurones either side of the median third ventricle. (D,E,F) X-ray film autoradiographs of the parvocellular AVT region scanned in experiment 1. (D) Control trout; (E) acutely confined trout; (F) repeatedly confined trout.

Experiment 1

Confinement significantly increased the AVT hybridization signal in the parvocellular neurones when these were monitored 2 h, 6 h or 26 h after the stress (Fig. 2). When the confinement stress was repeated daily over 6 days, however, the AVT signal was no longer significantly different from the controls. Plasma cortisol concentrations, which had declined after 2 h recovery from the stress, were again raised 4 h later (Fig. 2) indicating that the fish had experienced some additional, undefined, stress in the intervening period. Plasma

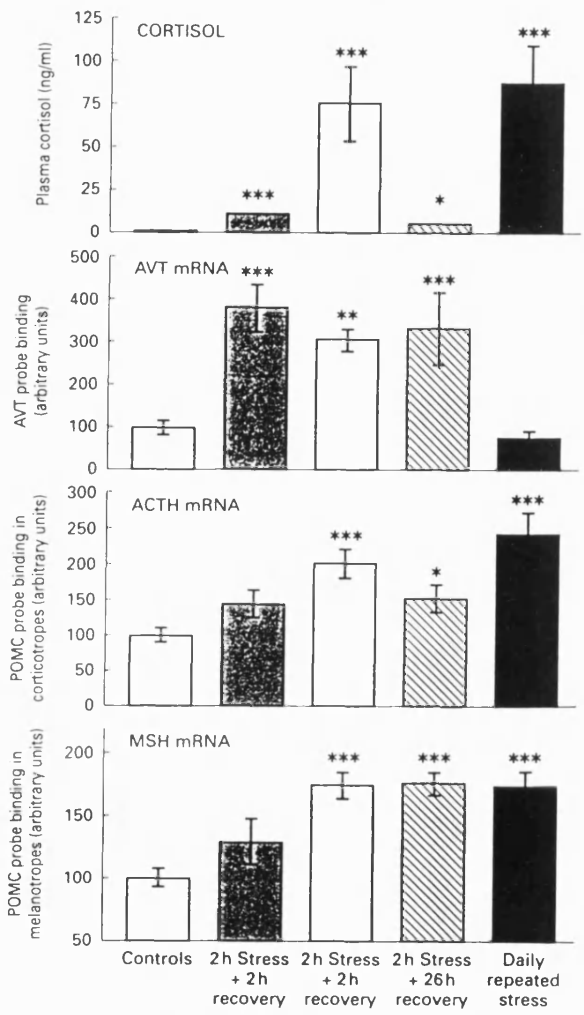


FIG. 2. Results of experiment 1. Trout were given either a single 2 h confinement stress followed by 2 h, 6 h or 26 h of recovery (columns 1–4), or six daily confinement episodes (column 5). The histograms show relative changes in plasma cortisol concentrations, arginine vasotocin (AVT) probe binding in the parvocellular neurones, or pro-opiomelanocortin (POMC) probe binding in the corticotropes and melanotropes. Statistical difference from controls: *$P<0.05$; **$P<0.01$; ***$P<0.001$ as determined by ANOVA followed by Fisher's LSD test. ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone.

cortisol concentrations were also very high in fish experiencing daily stress. Based on these results, fish in subsequent experiments were sacrificed 4 h after the start of the stress period.

Experiment 2 (acute stress)

The results of this experiment confirmed the ability of a single 2 h confinement stress to significantly increase the AVT hybridization signal (Fig. 3). In contrast, the stress of capture and transfer to low water for only 2 min had no significant effect on the AVT hybridization signal 4 h later (Fig. 3).

Experiment 3 (repeated stress)

Coincident with experiment 2, other fish from the same stock tank were subjected to repeated, daily stress (either confinement or low water) for 5 days. This repeated treatment elicited a rise in plasma cortisol concentrations similar to that seen after acute stress (Fig. 3) but, for both types of stress, the AVT hybridization signal was significantly lower than in control fish.

AVT hybridization signal in magnocellular neurones

No change in AVT hybridization signal was apparent in these neurones during either chronic or acute confinement stress, nor during the low-water stress (results not shown).

The effect of stress on POMC hybridization signal

Following an acute confinement stress in experiment 1, POMC hybridization signal was not significantly changed 2 h after the end of the stress (Fig. 2) but a significant increase was evident in both corticotropes and melanotropes at all later periods, including after repeated stress. The ability of repeated confinement stress to enhance the POMC transcripts in the corticotropes was confirmed by experiment 3 (Fig. 3) but a change in POMC transcripts in the melanotropes was not evident.

Discussion

The results show, for the first time, that AVT gene expression in a group of parvocellular neurones of the preoptic nucleus can be upregulated by a single 2-h period of confinement. This adds support to the idea that this region is homologous to the parvocellular neurones of the mammalian paraventricular nucleus. Previously, such homology was based on the responsiveness of these neurones in fish to treatments such as chemical adrenalectomy (23).

The upregulation of the AVT transcripts in these parvocellular neurones contrasts with the apparent lack of response by the magnocellular neurones, which are the probable homologues of the mammalian magnocellular paraventricular nucleus. In trout, the AVT transcripts in these magnocellular perikarya downregulate during adaptation to seawater (20), at which time circulating AVT levels are also depressed (24, 25) while plasma cortisol titres are initially raised (26). This response to seawater is in accordance with the possible osmoregulatory and vasoconstrictor role of AVT and may reflect a functional distinction between the two AVT neuronal groups.

It is presumed that an increase in AVT gene expression will lead to increased AVT peptide transcription for the purpose of maintaining a high level of ACTH secretion, or at least replacing the neuropeptide released during stress. We did not attempt to measure changes in AVT peptide levels in the hypothalamus partly because changes in secretory activity of the neurones confound peptide quantification, and also because transcriptional changes apparently affect only a

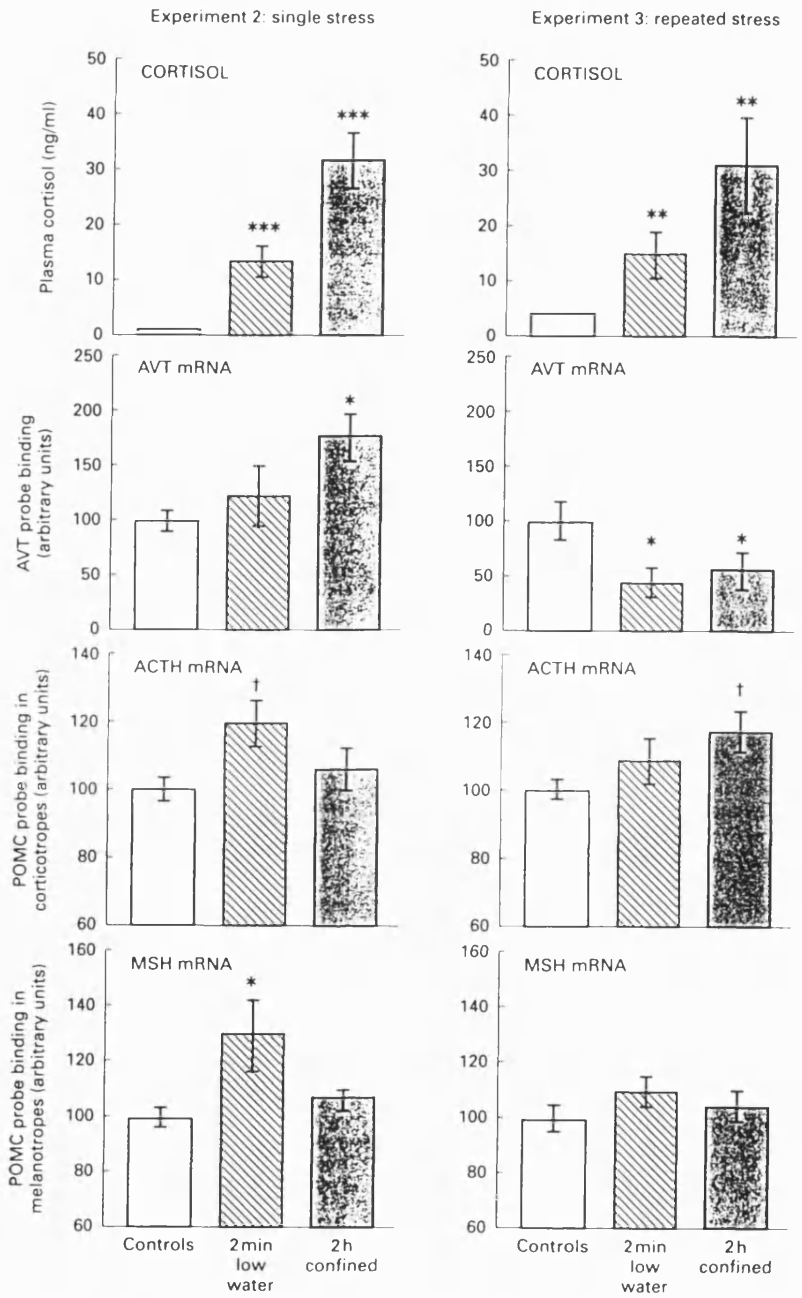


FIG. 3. Results of experiment 2 and experiment 3. In experiment 2, trout were stressed once by either capture and 2 min low water, or by 2 h confinement. Both groups were sacrificed 4 h after the start of stress. In experiment 3, trout were given either of these stresses daily over 5 days, and sacrificed 4 h after the start of the last stress. The histograms show relative changes in plasma cortisol concentration, arginine vasotocin (AVT) probe binding in the parvocellular neurones, or pro-opiomelanocortin (POMC) probe binding in the corticotropes and melanotropes, resulting from these treatments.Statistical difference from controls $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ as determined by ANOVA followed by Fisher's LSD test. †$P<0.05$ by Mann Whitney's U-test. ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone.

subpopulation of the AVT neurones, which cannot be dissociated for peptide measurements. We have previously found no change in hypothalamic AVT peptide concentration during the circadian cycle when, as in the present study, only the parvocellular neurones show significant changes in gene expression (19).

We have conducted additional experiments involving acute confinement stress but upregulation of the parvocellular AVT hybridization signal was not always significant. Reasons for this were wide individual variation resulting in a large standard error, an insufficiently intense stress (as judged by plasma cortisol concentrations) or an insufficently prolonged stress. Recent experiments suggest that mRNA may decline relatively rapidly after the end of stress (unpublished results). This might partly explain the failure to detect a change in AVT transcripts 4 h after brief low-water stress—the time lapse involved before a stress-induced increase in cytoplasmic CRF mRNA become significant in rats. Alternatively, it has been suggested that a brief stress might raise plasma cortisol concentrations without involving the hypothalamus. Thus, although confinement is known to induce ACTH release in the trout (27, 28), studies on the sea-bream suggest that 3 min withdrawal from water cause a very significant rise in plasma cortisol without significantly stimulating ACTH release (29). The authors therefore suggested that cortisol secretion in this case might be attributable to direct adrenergic or cholinergic stimulation of the steroidogenic tissue rather than activation of the hypothalamo-pituitary axis. Not having measured plasma ACTH, we cannot say if this was the case for the trout following low-water stress although it would be at odds with the apparent increase in POMC transcripts in the corticotropes.

Despite the ability of acute confinement to enhance AVT gene expression, the rise in transcripts did not persist, or was even depressed relative to controls, when the stress was repeated over several days. Even the milder stress of capture and 2 min low water, which had no acute effect, depressed the abundance of AVT gene transcripts when repeated, suggesting habituation to stress at some point along the neuronal pathway to the preoptic nucleus. Such habituation to certain stresses is well known to affect CRF transcripts in mammals (8, 30). It has been suggested that whether or not a stress induces habituation might depend on whether it is psychological or physiological in nature, these different stresses reaching the paraventricular nucleus through different pathways (31, 32). Certainly, the stresses employed in the present work (confinement or transfer to low water in which the fish are unable to maintain equilibrium) may be regarded as more psychological than physiological, and thus resemble the mammalian pattern by causing habituation.

Only changes in AVT transcription have been monitored in the present study but ACTH release is regulated by additional neuropeptides, including CRF and possibly also urotensin-I recently characterized from the trout hypothalamus (33, 34) and capable of stimulating ACTH release from goldfish pituitaries *in vitro* (16). During the course of persistant or repeated stress, it is probable that there is a change in the relative contribution of these peptides towards corticotrope stimulation, as is well documented in mammals. Thus, although AVT gene expression in the trout declines following repeated stress, this may not be the case for either CRF or urotensin-I, one or either of which could then be responsible for the increased abundance of POMC gene transcripts in the pituitary.

Acknowledgements

We thank the BBSRC for financial support of this work and Chris Davey, Mike Skinner and Ray Dickson for their help with fish care.

Accepted 23 February 2000

References

1 Herman JP, Schafer MKH, Sladek CD, Day R, Young EA, Akil H, Watson SJ. Chronic electroconvulsive shock-treatment elicits up-regulation of CRF and AVP messenger-RNA in select populations of neuroendocrine neurons. *Brain Res* 1989; **501:** 235–246.

2 Bartanusz V, Jezova D, Bertini LT, Tilders FJH, Aubrey JM, Kiss JZ. Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons. *Endocrinology* 1993; **132:** 895–902.

3 Aubry JM, Bartanusz V, Jezova D, Belin D, Kiss JZ. Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurones, but repeated stress is required to modify AVP immunoreactivity. *J Neuroendocrinol* 1999; **11:** 377–384.

4 Ma X-M, Levy A, Lightman S. Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *J Endocrinol* 1997; **152:** 81–89.

5 Darlingon DN, Barraclough CA, Gann DS. Hypotensive hemorrhage elevates corticotropin-releasing hormone messenger ribonucleic acid (messenger RNA) but not vasopressin messenger RNA in the rat hypothalamus. *Endocrinology* 1992; **130:** 1281–1288.

6 Kjaer A, Larsen PJ, Knigge U, Jorgensen H, Wargerg J. Neuronal histamine and expression of corticotrophin-releasing hormone, vasopressin and oxytocin in the hypothalamus: relative importance of H-1 and H-2 receptors. *Europ J Endocrinol* 1998; **139:** 238–243.

7 Harbuz MS, Ress RG, Eckland D, Jessop DS, Brewerton D, Lightmann SL. Paradoxical response of CRF mRNA and CRF-41 peptide and adenohypophyseal POMC mRNA during chronic inflammatory stress. *Endocrinology* 1992; **130:** 1394–1400.

8 Hatalski CG, Guirguis C, Baram TZ. Corticotropin-releasing factor mRNA expression in the hypothalamic paraventricular nucleus and the central nucleus of the amygdala is modulated by repeated acute stress in the immature rat. *J Neuroendocrinol* 1998; **10:** 663–670.

9 Van den Dungen H, Buijs R, Pool C, Terlou M. The distribution of vasotocin and isotocin in the brain of the rainbow trout. *J Comp Neurol* 1982; **212:** 146–157.

10 Olivereau M, Olivereau J. Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. *Peptides* 1988; **9:** 13–21.

11 Olivereau M, Moons L, Olivereau J, Vandersande F. Coexistence of corticotropin-releasing factor-like immunoreactivity and vasotocin in perikarya of the preoptic nucleus in the eel. *Gen Comp Endocrinol* 1988; **70:** 41–48.

12 Yulis C, Lederis K. Co-localization of the immunoreactivities of corticotropin-releasing factor and arginine vasotocin in the brain and pituitary system of the teleost *Catastomus commersoni*. *Cell Tiss Res* 1987; **247:** 267–273.

13 Batten TFC, Cambre ML, Moons L, Vandesande F. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna*. *J Comp Neurol* 1990; **302:** 893–919.

14 Okawara Y, Ko D, Morley SD, Richter D, Lederis KP. *In situ* hybridization of corticotropin-releasing factor-encoding messenger RNA in the hypothalamus of the white sucker, *Catastomus commersoni*. *Cell Tiss Res* 1992; **267:** 545–549.

15 Holmqvist B, Ekstrom P. Hypophysiotrophic systems in the brain of the atlantic salmon. Neuronal innervation of the pituitary and the origin of pituitary dopamine and nonapeptides identified by means of combined carbocyanine tract tracing and immunocytochemistry. *J Chem Neuroanat* 1995; **8:** 125–145.

16 Fryer J, Lederis K, Rivier J. ACTH-releasing activity of urotensin I and ovine CRF: interactions with arginine vasotocin, isotocin and arginine vasopressin. *Reg Peptides* 1985; **11:** 11–15.
17 Baker BI, Bird DJ, Buckingham JC. In the trout, CRH and AVT synergize to stimulate ACTH release. *Reg Peptides* 1996; **67:** 207–210.
18 Pierson PM, Guibollini ME, Lahlou B. A V-1 type receptor for mediating the neurohypophysial hormone-induced ACTH release in trout pituitary. *J Endocrinol* 1996; **149:** 109–115.
19 Gilchriest BJ, Tipping DR, Levy A, Baker BI. Diurnal changes in the expression of genes encoding for arginine vasotocin and pituitary pro-opiomelanocortin in the rainbow trout (*Oncorhynchus mykiss*): correlation with changes in plasma hormones. *J Neuroendocrinol* 1998; **10:** 937–943.
20 Hyodo S, Urano A. Changes in expression of provasotocin and proisotocin genes during adaptation to hyper- and hypo-osmotic environments in rainbow trout. *J Comp Physiol B* 1991; **161:** 549–556.
21 Salbert G, Chauveau I, Bonnec G, Valotaire Y, Jego P. One of the two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol Endocrinol* 1992; **6:** 1605–1613.
22 Suzuki M, Bennett P, Levy A, Baker BI. Expression of pro-MCH and POMC genes in rainbow trout (*Oncorhynchus mykiss*) during ontogeny and in response to early physiological changes. *Gen Comp Endocrinol* 1997; **107:** 341–350.
23 Olivereau M, Olivereau J. Effect of pharmacological adrenalectomy on corticotropin-releasing factor-like and arginine vasotocin immunoreactivities in the brain and pituitary of the eel: immunocytochemical study. *Gen Comp Endocrinol* 1990; **80:** 199–215.
24 Perrot M, Carrick S, Balment R. Pituitary and plasma arginine vasotocin levels in teleost fish. *Gen Comp Endocrinol* 1991; **83:** 68–74.
25 Kulczykowska E. Reponse of circulating arginine vasotocin and isotocin to rapid osmotic challenge in rainbow trout. *Comp Biochem Physiol A* 1997; **118:** 773–778.
26 Sakomoto T, Hirano T. Growth hormone receptors in the liver and osmoregulatory organs of the rainbow trout: characterization and dynamics during adaptation to seawater. *J Endocrinol* 1991; **130:** 425–433.
27 Sumpter JP, Dye HM, Benfey TJ. The effects of stress on plasma ACTH, MSH, and cortisol levels in salmonid fishes. *Gen Comp Endocrinol* 1986; **62:** 377–385.
28 Balm PHM, Pottinger TG. Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 1995; **98:** 279–288.
29 Ruane N, Wendelaar Bonga S, Balm P. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. *Gen Comp Endocrinol* 1999; **115:** 210–219.
30 Ma X-M, Levy A, Lightman S. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology* 1997; **138:** 4351–4357.
31 Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenal axis. *Trends Neurosci* 1997; **20:** 78–84.
32 Sawchenko P, Imaki T, Potter E, Kovacs K, Imaki J, Vale W. The functional neuroanatomy of corticotropin-releasing factor. *Ciba Foundation Symp* 1993; **172:** 5–29.
33 Barsyte D, Tipping DR, Smart D, Conlon JM, Baker BI, Lovejoy DA. Rainbow trout (*Oncorhynchus mykiss*) urotensin-I: structural differences between urotensins-I and urocortins. *Gen Comp Endocrinol* 1999; **115:** 169–177.
34 Lovejoy DA, Balment RJ. Evolution and physiology of the corticotrophin-releasing factor (CRF) family of neuropeptides in vertebrates. *Gen Comp Endocrinol* 1999; **115:** 1–22.

4.3 Additional Materials and Methods

In addition to the published observations, details of unpublished work relating to the expression of AVT, CRH, IT and MSH transcript responses to stress is given below.

4.3.1 Effect of acute and chronic stress on IT mRNA

In order to investigate the role of isotocin the stress response duplicate tissue samples from Expt 1 in the published paper, in which fish were subjected to a two hour confinement stress (56g/L) applied either singly or daily for five days, were hybridised with a ^{35}S-labelled isotocin deoxyribonucleotide probe.

4.3.2 Effects of acute stress on plasma cortisol, AVT, CRH and melanotrope POMC mRNAs

Fish were removed from the same 450L tank and stressed by confinement for different durations with or without periodic disturbance (see Table 4.1). Stress paradigms were as

Table 4.1; Details of experiments to test the effects of different acute stressors on plasma cortisol, AVT, CRH and melanotrope POMC mRNAs. Fish were either chased with a net, the bucket agitated, or fish briefly handled for one minute during disturbance (where applicable). For shallow water fish were transferred to a tank containing water to a depth of 30cms. CRH Trial = stressors applied for the purpose of obtaining tissue for CRH trial *in situ* hybridisations. Confinement density is given in parenthesise where applicable. n = number of fish per group.

Expt No.	n	Fish Weight (g±SEM)	Stress Paradigm	Disturbance frequency	Recovery Period
1	8	52±2	2hrs net confined (35g/L)	-	0, 2, & 6 hrs
2	6	308±10	4hrs confined (185g/L)	-	0
3	8	262±13	5hrs confined (118g/L)	Hourly	0
CRH	10	309±27	(a) 6hrs confined (119g/L)	Every 30 mins	0
Trials			(b) 6hrs shallow water	Every 30 mins	0

described in the published paper (Section 4.2) with the exception of net confinement in which fish were transferred to a net arranged such that animals were in constant contact with the net with just 2 cms of water above them. For shallow water, fish were transferred to a new tank containing water to a depth of 30cms. A steady flow through of well aerated water was maintained throughout the duration of the stresses. A recovery period, where employed, was carried out in home tank water. At the end of each experiment fish were anaesthetised, blood collected for cortisol radioimmunoassay, and brains and pituitaries removed for *in situ* hybridisation.

4.3.3 CRH *in situ* hybridisation trials

Since previous work (Ando *et al.*, 1999) found that CRH transcripts were only visible in trout stressed by confinement for three hours with disturbance, fish treated in a similar manner were used in all CRH trials (see Table 4.1). Since these were not designed for quantitative work no control fish were collected.

A number of different *in situ* hybridisation conditions were tested in order to try and determine a suitable and repeatable technique. Two different probes complimentary to the mature peptide (Probe 1), or a region encoding for the pre-CRH peptide (Probe 2) sequence in sockeye salmon (Ando *et al.*, 1999) were used. The sequence for each probe from 5' to 3' were as follows;

CRH Probe 1; `cat gtc gaa cgt aag atc tag aga tat cgg`

CRH Probe 2; `gt cgt cga gct ggt tcg cga agc aga`

The basic methodology was as described previously for both wax embedded and frozen tissue (see Section 2.5). Variations to this were made with respect to probe CPM per slide (between $3x10^4 \rightarrow 5x10^5$ CPM/slide), hybridisation temperature (37 and 42 °C), and washing temperature (50 → 60°C). Both wax and frozen sections were used. Full details of the conditions and results of all CRH *in situ* hybridisations carried out are given in Table A.1 of the Appendix.

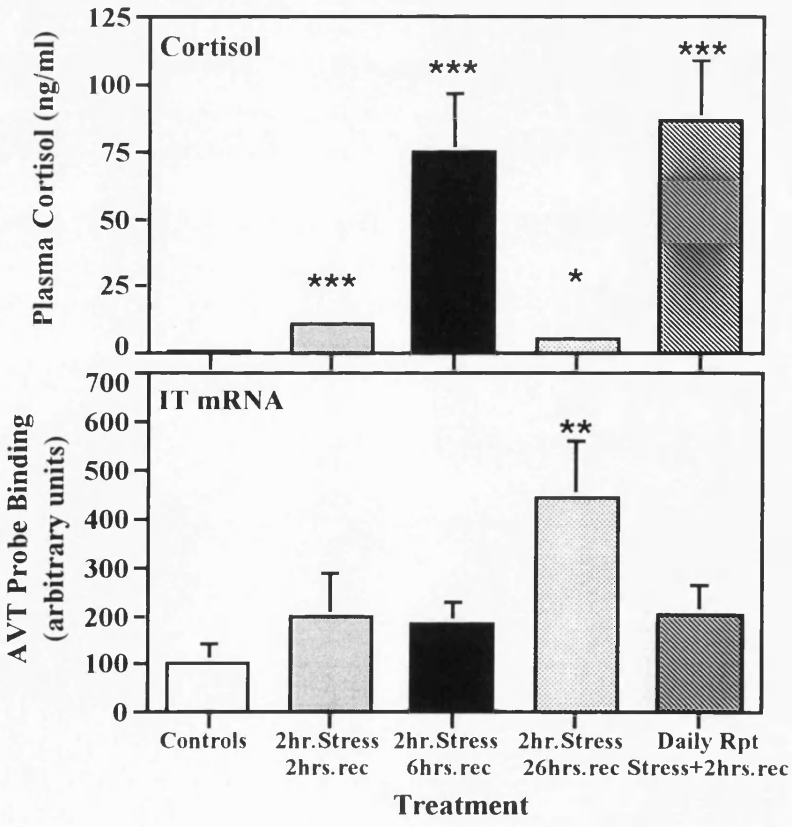
An additional set of brain tissue from the cerebellum region, not thought to contain CRH (as determined by immunocytochemistry), was hybridised as a negative control. An AVT hybridisation was carried out in tandem for positional comparison. Autoradiographic film was exposed for an initial period of six days. If no CRH signal was detectable this was extended to a maximum of four weeks.

4.4 Additional Results

4.4.1 Effect of acute and chronic stress on IT mRNA

Isotocin transcripts were significantly raised 26 hours after acute confinement stress (see Figure 4.1). Plasma cortisol titres in this same experiment were significantly raised following two hours recovery. Following six hours recovery cortisol titres increased further, indicating that the fish may have experienced a further unknown stress in the interim period. When the stressor was applied chronically plasma cortisol concentrations were significantly raised whilst IT transcripts remained at basal levels.

Figure 4.1; Response of isotocin mRNA to a two hour confinement stress and following 2, 6, or 26 hours recovery. Histograms are means ±SEM. Plasma cortisol results were as described in the published paper and have been included here for ease of comparison. Statistical difference from controls is displayed thus; * = P<0.05; ** = P<0.01; *** = P<0.001 as determined by one-way ANOVA followed by Fisher's LSD test. rec = recovery.

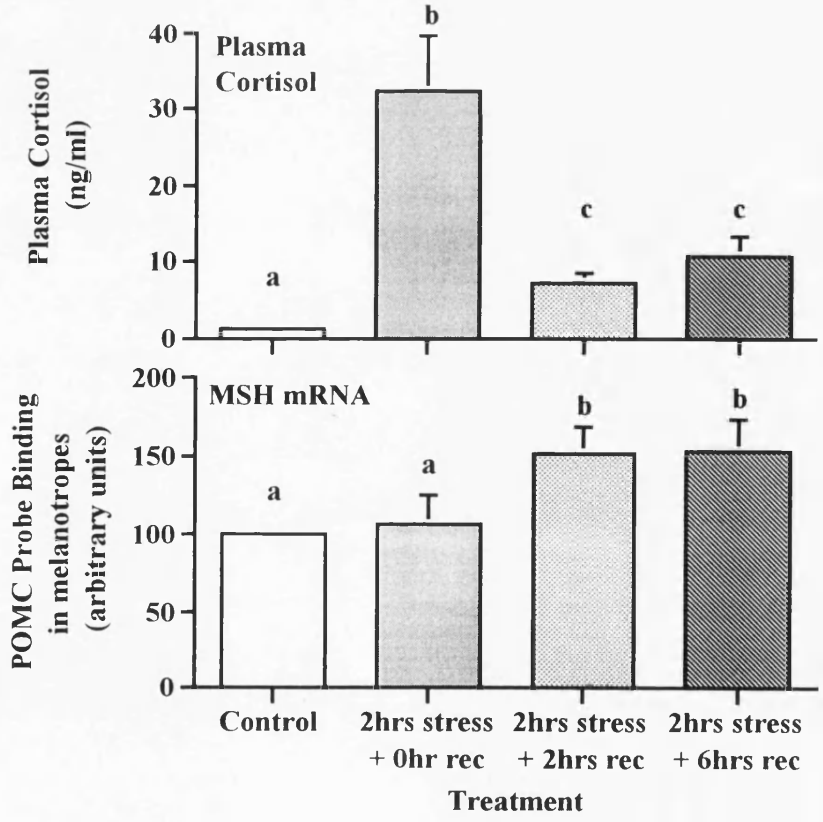


4.4.2 Effects of acute stress on plasma cortisol, AVT, CRH and melanotrope POMC mRNAs

Experiment 1

Plasma cortisol titres were significantly raised ($P<0.05$ one-way ANOVA) immediately following the stress, reaching 33±7ng/ml. Although concentrations declined after two or six hours of recovery they did not return to basal (see Figure 4.2). Interestingly POMC probe binding in the melanotropes was raised four hours after the start of the stress (see Figure 4.2), whilst corticotrope POMC was unaffected by the stress (results not shown).

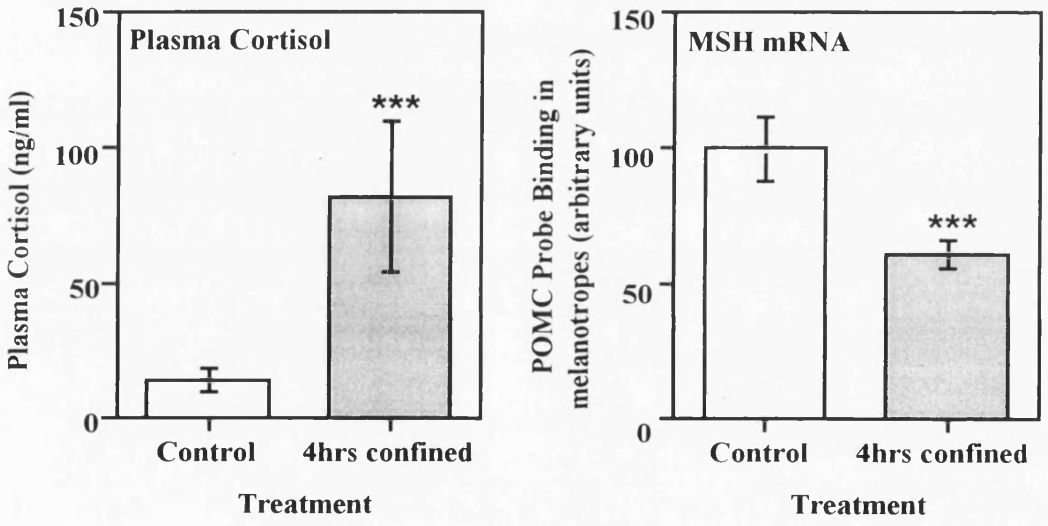
Figure 4.2; Results from Expt 1. Fish were stressed with two hours net confinement followed by 0, 2, or 6 hours of recovery (rec). Graphs show changes in plasma cortisol and POMC probe binding in the pituitary melanotropes. Bars are means ± SEM. Superscripts were assigned using Fisher's LSD Test following one-way ANOVA ($P<0.05$); bars with different superscripts are significantly different from one another.



Experiment 2

When sampled immediately after the stress cortisol titres attained 82±28ng/ml, this being a clear significant rise above control levels. As in Expt 1, POMC levels in the corticotropes were unaffected by the stress (results not shown). However, in contrast to the response seen in Expt 1, POMC probe binding in the melanotropes declined to 61% of control values (see Figure 4.3).

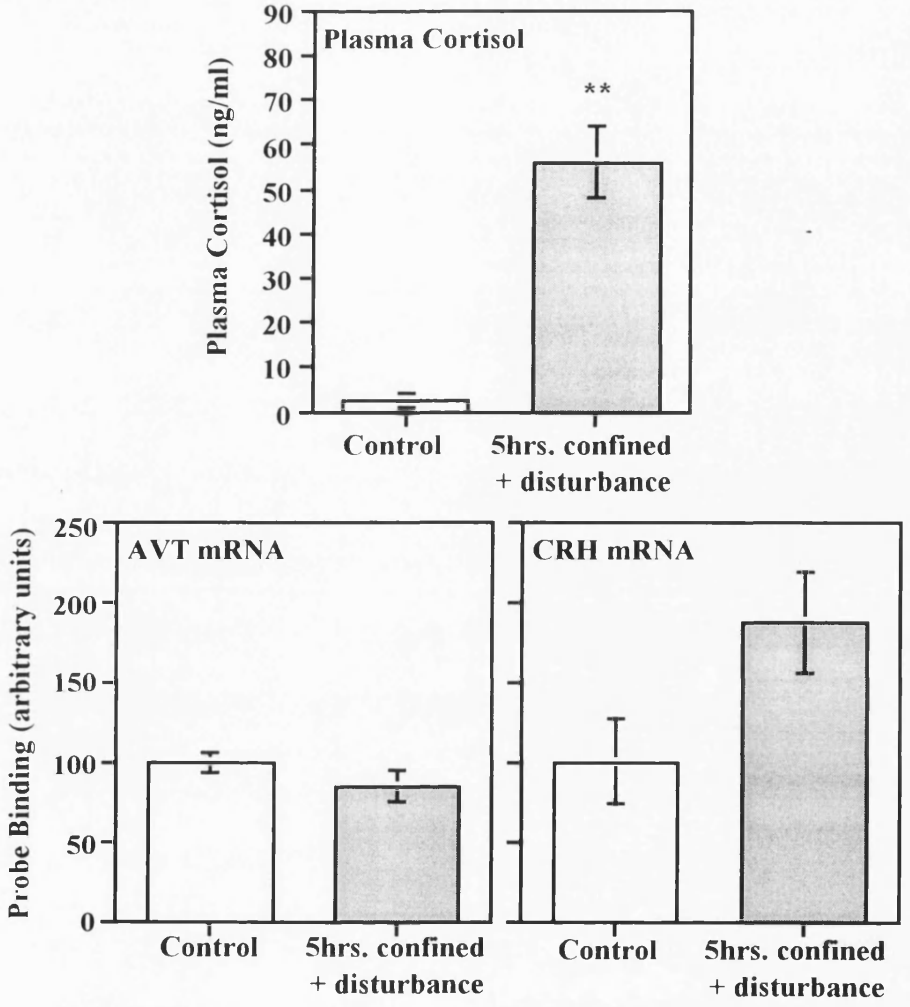
Figure 4.3; Results from Expt 2. Fish were stressed with four hours confinement and sampled without recovery. Graphs show changes in plasma cortisol and POMC probe binding in the pituitary melanotropes. Bars are means ± SEM. *** = P<0.01, one-way ANOVA as compared to control.



Experiment 3

As in all prior experiments plasma cortisol titres were significantly raised by the confinement stress (see Figure 4.4). Since the distribution pattern of CRH matched that of AVT the same region of cells was scanned as described in Section 2.5.6. Transcript levels in the parvocellular AVT neurones were unaffected by the stress (see Figure 4.4). However, those in the CRH parvocellular perikarya were increased, reaching 187% of control levels. This rise in CRH mRNA was not, however, significant (p=0.07, one-way ANOVA).

Figure 4.4: Results from Expt 3. Fish were stressed with five hours confinement with hourly disturbance and sampled without recovery. Graphs show plasma cortisol, and AVT and CRH mRNAs. Bars are means (± SEM). ** = P<0.01 (one-way ANOVA) as compared to control.



4.4.3 CRH *in situ* hybridisation trials

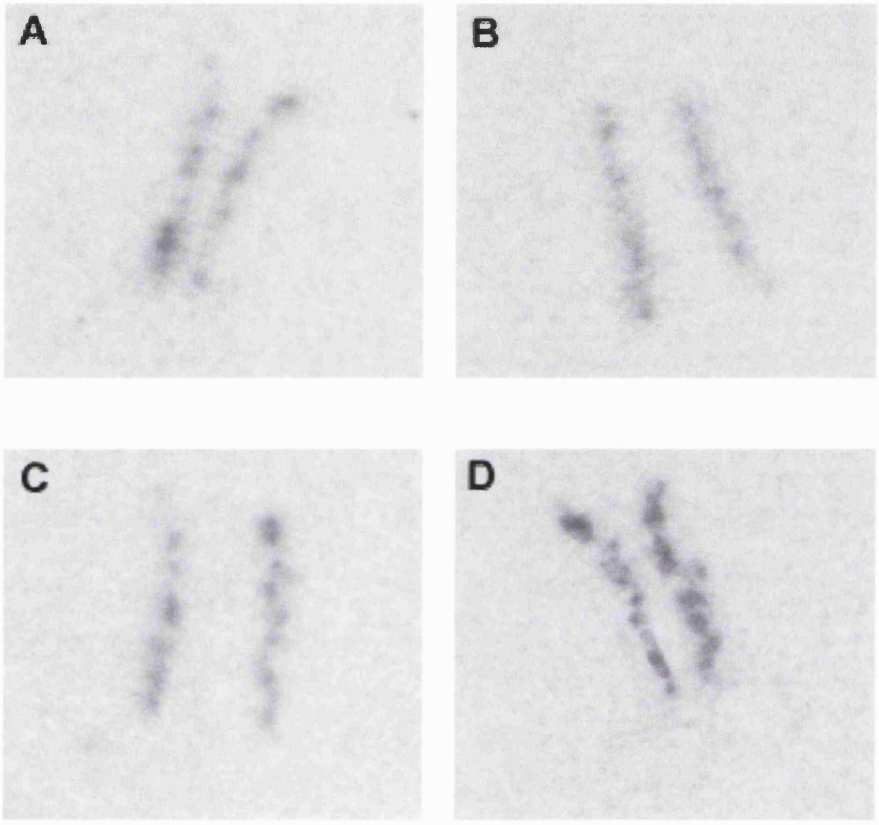
Positive CRH signals were obtained using both wax and frozen sections under a variety of conditions (see Table 4.2) though only whilst using Probe 1. However, even when such conditions were duplicated this was not always repeatable. An extension of autoradiograph exposure to four weeks failed to visualise CRH mRNA-positives if none were present following a six day exposure. All AVT positive controls producing clearly visible autoradiograph signal, with none visible in negative controls.

Table 4.2; *In situ* hybridisation conditions under which a CRH mRNA-positive signal was obtained on autoradiographic film. Exposure time was six days in all cases. Activity/100µl is the final CPM (counts per minute) of the probe in 100µl of hybridisation mixture. Hybridisation and washing temperatures are quoted in °C.

Tissue Treatment	Activity/ 100µl	Hybridisation Temperature	Washing Temp.
Frozen	$5x10^5$	37	60
Wax	$5x10^5$	42	51.5
Wax	$5x10^5$	42	55
Wax	$5x10^5$	37	51.5
Wax	$5x10^5$	37	55

CRH probe binding, where visible, was in the pre-optic region on either side of the 3rd ventricle (see Figure 4.5, overleaf) and matched that seen following immunocytochemistry (Olivereau & Olivereau, 1988*a*, 1990*a*; Gilchriest *et al.*, 1998). The distribution pattern overlapped with that of AVT probe binding in the parvocellular region. The full extent of probe binding in the magnocellular region was not investigated.

Figure 4.5; Image capture of autoradiographic film showing CRH mRNA probe binding in parvocellular neurones of the preoptic nucleus of rainbow trout. Fish were stressed by (A) six hours confinement with hourly disturbance, or (B) shallow water with hourly disturbance for six hours (CRH Trials). (C) and (D) are from control and stressed animals (confinement plus half hourly disturbance) respectively from Expt 3. All sections are from the same region as that scanned for parvo-AVT.



4.5 Additional Discussion

In order to put the additional discussion into context the main issues already raised by the published paper are summarised here. The published results show for the first time that AVT transcripts in the parvocellular pre-optic region can be upregulated by a single two hour confinement stress. However, this upregulation is not a consistent response. It is thought that the lack of response to some acute stressors could be due to large variability between individuals, an insufficiently intense stress (as deemed by plasma cortisol titres), or an insufficiently prolonged stress. It has also been suggested that stimulation of cortisol secretion can occur independently of the hypothalamo-pituitary axis via direct adrenergic or cholinergic stimulation of steroidogenesis (Ruane *et al.*, 1999). In such instances AVT transcripts would be unaffected. When either a mild (two minute low water) or severe (two hour confinement) stress was applied repeatedly for five or six days AVT gene transcripts declined to, or below, basal levels. This is presumed to be due to habituation or reflects an upregulation of other ACTH secretogogues such as CRH or urotensin-1. Although the magnocellular AVT neurones did not respond to any of the stressors tested this could reflect a functional difference between the two cell types since the magnocellular neurones are implicated more closely with the control of osmoregulation (Perrot *et al.*, 1991; Kulczykowska, 1997).

4.5.1 The response of CRH mRNA to stress and the problems with probe binding

Although parvocellular CRH transcripts appear to rise in the absence of an AVT mRNA response this increase was not significant. It is unfortunate that a reliable *in situ* hybridisation methodology could not be determined during the course of this study since this apparent dissociation between the response of CRH and AVT mRNAs is of considerable interest. In the rat such independent control of CRH and AVP has been widely demonstrated (Harbuz & Lightman, 1992) and is believed to be of particular importance in the response to chronic stress (De Goeij *et al.*, 1991, 1992; Harbuz *et al.*, 1992) in which AVP plays a more central role than CRH (Ma *et al.*, 1997*a*). Whether this occurs in fish would require both the determination of a reliable CRH *in situ* hybridisation method and additional investigation into the transcript response of both AVT and CRH to acute and chronic stress. Such studies may also serve to clarify the

lack of an AVT mRNA response in some instances; it is possible that an upregulation of CRH is maintaining ACTH secretion in the absence of an AVT mRNA response.

The rationale behind the problems experienced with CRH *in situ* hybridisations in the current study are difficult to determine. Use of immunocytochemistry has demonstrated ample CRH peptide within the PON of rainbow trout, amongst other teleost species (Olivereau & Olivereau, 1988*a*, 1990; Olivereau *et al.*, 1988; Matz & Hofeldt, 1999; Zupanc *et al.*, 1999) though the correlation between peptide quantity and transcripts is poor in the white sucker (Okawara *et al.*, 1992). This is in contrast to AVT and IT, in which both abundant peptide (Van den Dungen *et al.*, 1982) and mRNA have been localised in the brain of trout, with a closely matching distribution pattern (Hyodo & Urano, 1991). The reasons for the poor correlation between CRH peptide and its mRNA are unclear, though given the difficulties with the visualisation of CRH transcripts it is possible that this is related to problems with detection as opposed to a lack of mRNA present. A diverse range of techniques have been employed in order to overcome this problem in other studies on teleosts. Although successful these methods, including the use of ^{32}P (Okawara *et al.*, 1992; Bernier *et al.*, 1999) or digoxigenin labelled oligoprobes (Ando *et al.*, 1999), emulsion dipping (Okawara *et al.*, 1992) and Northern blot (Bernier *et al.*, 1999), are impractical for use with large experiments, such as used in the current study, or incompatible with the techniques required to quantify the visible signal.

4.5.2 The variability in the AVT transcript response to acute stress

In the current study we concentrated on sampling fish four hours after the start of the stress, since this is the optimal time required for both CRH and AVP mRNAs to be significantly raised in the rat (Lightman & Young, 1989; Ma *et al.*, 1997*b*). Indeed, initial work on stress, detailed in the published paper, shows that AVT transcripts are elevated both four and eight hours after the start of the stress. However, recent work in the flounder has shown that parvocellular AVT mRNA is largely unaffected when measured three hours after a 30 minute confinement, and is not significantly raised until 24 hours after the start of the stress (Bond *et al.*, 2000). The absence of a visible AVT mRNA response in some experiments may be due to such temporal differences, with changes in AVT transcripts missed as a result of the sample times used.

Immunoreactive AVT fibres are found in both the pars intermedia and the pars distalis of the pituitary which may originate in either, or both, the parvo- and magno- cellular neurones of the PON. The latter of these are implicated in the control of osmoregulation and are thought to terminate in the pars intermedia where they release their peptide into the blood. In rainbow trout adapted to freshwater circulating plasma AVT concentrations are approximately 65% higher than those of fish adapted to brackish water (Kulczykowska, 1997). Interestingly, however, upon transfer from fresh to brackish water plasma AVT briefly rises; presumably this is a response to the transfer stress. A similar transitory increase in circulating AVT concentration is seen in eels transferred from fresh- to sea- water (Balment *et al.*, 1993). If, as previously thought, the parvocellular neurones consist of a heterogeneous population and thus contribute to circulating AVT then changes in parvocellular transcripts may reflect changes in peptide release to both pituitary cell types. In such an instance finer changes which may occur in those parvocellular perikarya which influence corticotropic activity would not be seen unless they were coupled with a change in release into the plasma.

Stressors which do not upregulate AVT transcripts could be acting to stimulate transcription of other ACTH secretogogues such as CRH, urotensin-1, or isotocin in order to maintain cortisol secretion. Indeed, results suggest that when AVT mRNA does not respond to an intense confinement, CRH transcripts increase. Further investigation is required to clarify this. Urotensin-1 has been shown to stimulate ACTH release in the goldfish (Fryer *et al.*, 1985) and elevates cortisol secretion in trout (Arnold-Reed & Balment, 1994) and flounder (Kelsall & Balment, 1995) isolated interrenal preparations. This neuropeptide has also recently been characterised from trout hypothalami (Barstye *et al.*, 1999); thus, hypothalamic urotensin could be acting on the pituitary corticotropes or directly on the interrenal. The importance of this neuropeptide in the control of stress is little understood at present. However, low levels of expression in the goldfish brain relative to that of CRH (Bernier *et al.*, 1999) suggest that the importance of urotensin-1 as an ACTH-secretogogue, as compared to CRH, may be low, at least in this species.

The upregulation of IT transcripts in the current study are difficult to interpret in themselves since plasma cortisol results indicate that an additional and undefined stress may have acted to raise transcripts at the 26 hour recovery time point. Its weak effects on ACTH secretion in the trout (Pierson *et al.*, 1996) suggests that it has a lesser function in

stress-induced ACTH secretion as compared to either AVT or CRH, though this requires further study.

All eggs were obtained from the same supplier and genetic stock in each successive year. Thus, genetic variations in the responsiveness to stress, which shows a moderately high heritability for the confinement-induced plasma cortisol response (Pottinger & Carrick, 1999), is unlikely to account for the lack of repeatable AVT mRNA increases following stress. Indeed, the consistency of supply also discounts possible differences in HPI responsiveness that occurs between different strains of rainbow trout (Pottinger & Moran, 1993).

Following maturation the amount of ACTH released by trout in response to stress is reduced, presumably reflecting a desensitisation of pituitary corticotropes to AVT/CRH or a reduction in the release of these ACTH-secretogogues (Pottinger *et al.*, 1995). However, such changes are unlikely to account for any variations in the response to stress in our animals since all fish used were under 18 months of age and therefore immature.

4.5.3 Habituation and the response of transcripts to chronic stress

Although a two hour confinement enhanced AVT gene transcription when applied once, the rise in transcripts did not persist when the stress was repeated on a daily basis. Even the low water stress, which had no effect when administered acutely, reduced AVT mRNA levels when applied chronically. An analogous shift in the effects of acute versus chronic stress has been observed in the rat. Thus, acute confinement will increase CRH mRNA in the parvocellular PVN (Ma *et al.*, 1997*b*). However, when applied daily for two weeks the CRH hybridisation signal is unaffected, remaining at control levels (Ma *et al.*, 1997*a*).

Various studies carried out on the rat indicate that whether such habituation occurs or not may be due to the relative psychological or physiological components of the stressor (Herman *et al.*, 1996; Herman & Cullinan, 1997; Hatalski *et al.*, 1998). Thus, stressors which present an immediate physiological threat, such as injection of hypertonic saline, are perceived by pathways which reach the PVN via the brain stem, whereas repeated psychological stressors reach the forebrain via higher brain structures (Herman &

Cullinan, 1997). These multiple neuronal inputs to the CRH and AVP neurones of the PVN have been demonstrated in the rat in a number of studies (Sawchenko *et al.*, 1993; Herman *et al.*, 1996), and it is possible that such different pathways by which a stress is perceived could be present in fish. Indeed those stressors used in the current study which invoke habituation (i.e. confinement and low water) could be regarded as psychological stressors, and thus resemble the mammalian pattern in causing habituation. In the rat, however, where habituation occurs CRH is downregulated whilst AVP mRNA expression is maintained at high levels. In the trout, it appears to be the reverse, with AVT, the homologue of AVP, becoming habituated to the stress. Whether CRH transcripts are up-regulated in such an instance remains to be tested.

4.5.4 The reaction of pituitary melanotrope POMC transcripts to acute stress

The response of POMC mRNA in the pituitary melanotropes, which presumably reflects changes in release, varies between experiments. Thus, following confinement transcripts may show no change, decline, or increase. Such disparities in the response of α-MSH to stress in fish has been previously reported. Thus, in the brown trout, confinement alone may have no affect (Sumpter *et al.*, 1985, 1986; Pickering *et al.*, 1986) or cause a decline (Balm & Pottinger, 1995) in circulating concentrations of α-MSH. However, when confinement is combined with either thermal shock (Sumpter *et al.*, 1985; Pickering *et al.*, 1986) or restraint (Sumpter *et al.*, 1986) α-MSH release is up-regulated. Intense noise stress will also increase circulating α-MSH concentrations when applied for one hour, though not when applied for 24 hours (Gilham & Baker, 1985). The administration of exogenous cortisol suggests that these variations in responsiveness may be due to a sensitivity of the melanotropes to corticosteroid negative feedback (Balm *et al.*, 1993; Balm & Pottinger, 1995).

That α-MSH is upregulated in response to stress in the absence of an associated rise in ACTH, as indicated by POMC transcripts in the current study, has been previously reported. In the marine teleost, *Boops salpa*, noise stress stimulates synthesis and secretion from the melanotropes but not corticotropes (Malo-Michele, 1980), whilst a three minute confinement induces plasma increases in both cortisol and α-MSH but not ACTH in the sea bream (Arends *et al.*, 1999). Taken together it would seem that α-MSH is stimulating cortisol release, however, α-MSH's relatively low corticosteroidogenic

potency indicates this is unlikely to be the case (Rance & Baker, 1981). *In vitro* evidence suggests that the control of cortisol is multi-factorial and potentially involves endorphin, α-MSH, urotensin-1, and angiotensin-II (Arnold-Reed & Balment, 1994; Balm & Pottinger., 1995) as well as ACTH. The recent discovery of novel POMC-derived peptides (Salbert *et al.*, 1992; Chauveau *et al.*, 1993) may also be of relevance. It appears that the control of pituitary mediated cortisol release is likely to be a combination of these different inputs though this requires additional investigation. The role of α-MSH within this context continues to be difficult to define.

4.5.5 Summary

The upregulation of AVT mRNA in the parvocellular neurones of the pre-optic nucleus following acute stress lends support to the proposed role of this neuropeptide in the stress response. That this does not occur consistently is likely to be due to a combination of influences, though could also be related to sample time. The downregulation of transcripts following chronic stress suggests AVT may be acting as CRH in rats, whereby there is a shift in the dominant ACTH-secretogogue, though this would require additional investigation in order to confirm.

Chapter 5

Sensitivity of AVT and POMC mRNAs to Cortisol Negative Feedback

5.1 Introduction

In fish the HPI axis is activated following stress, resulting in the release of cortisol from the interrenal tissue. Corticosteroid released from the adrenal tissue in response to ACTH modulates its own release by completing a negative feedback loop at the pituitary and hypothalamic level. The circulating concentration of this steroid has a significant influence on the co-ordination of the stress-induced activity of the HPI axis as it acts to contain the pathophysiological responses to stress. An understanding of its role in this context is therefore critical.

In the rat, this feedback effect on the HPA has been demonstrated by a number of methodologies. The removal of endogenous corticosterone by pharmacological adrenalectomy results in increases in both release (Plotsky & Sawchenko, 1987) and transcription (Wolfson *et al.*, 1985; Davis *et al.*, 1986; Lightman & Young, 1989; Swanson & Simmons, 1989) of AVP and CRH, and an increase in the number of AVP/CRH co-localising neurones (Kiss *et al.*, 1984; Sawchenko *et al.*, 1984). Presumably this is due to the removal of corticosterone inhibition on these neurones. Administration of synthetic corticosteroids, such as dexamethasone, will also reduce CRH mRNA in intact rats (Harbuz & Lightman, 1989*a*). AVP, however, is less sensitive to this inhibition (Antoni, 1986; Wand & Eipper, 1987; Levin & Roberts, 1991; Aguilera *et al.*, 1992). Since there is an increased requirement for AVP during chronic stress (see Section 1.3.1) when plasma corticosterone levels are elevated for long periods, a reduced sensitivity to corticosteroid negative feedback would act to facilitate this requirement under such conditions. The pituitary corticotropes are also known to be sites at which glucocorticoids have inhibitory actions on both synthesis and secretion of ACTH (Sayers & Portanova, 1974; Widmaier & Dallman, 1984). Thus, at the pituitary level adrenalectomy results in a marked increase in both ACTH transcription, synthesis, and secretion, which are reversible or prevented by treatment with glucocorticoids (Dallman *et al.*, 1974; Birnberg *et al.*, 1983; Eberwine & Roberts, 1984; Tannahill, 1987).

There appear to be three distinct molecular mechanisms of negative feedback which are effective over different time domains (Buckingham, 1996). These are defined as 'rapid', 'early delayed', and 'late delayed' phases (Keller-Wood & Dallman, 1984; Buckingham

et al., 1992). The 'rapid' phase is sensitive to the rate of change rather the absolute concentration of circulating glucocorticoid and responds quickly, though transiently (under 15 mins.); this is followed by the 'early delayed' phase detectable some thirty minutes to two hours later which may persist for up to 24 hours (Buckingham *et al.*, 1992). The final stage, 'late delayed', is usually only manifest following substantial rises in glucocoticoids that follow chronic stress or treatment with high doses of exogenous steroids (Buckingham, 1996).

There is some evidence to indicate an extra-hypothalamic component to the negative feedback effect since relatively low levels of corticosterone do not appear to act directly on the CRH neurones (Bradbury *et al.*, 1991). This is perhaps not surprising since negative feedback exerted at a site proximal or in parallel to the final neuroendocrine output neurone allows for far greater flexibility and adaptability than if the feedback acted directly to inhibit the CRH neuronal motor that powers the system (Dallman, 1993). At the cellular level, lipocortin 1 (LC1), expressed in both the pituitary (Christian *et al.*, 1997) and PVN (Taylor *et al.*, 1997), is a key mediator of the glucocorticoid action. The release of LC1 is stimulated by glucocorticoids (Flower, 1988) whereupon it exerts a paracrine/autocrine inhibitory action on peptide release (Buckingham, 1996; Christian *et al.*, 1996).

In the rat there are two glucocorticoid receptor types, mineralocorticoid (MR) and glucocorticoid (GR) receptors (Harbuz & Lightman, 1992), both of which are implicated in the corticosterone-mediated negative feedback control over the HPA (Murphy *et al.*, 1998). In fish the negative feedback mechanism is slightly simplified in that the system has only one type of cortisol receptor (glucocorticoid type; Ducouret *et al.*, 1995). These receptors have been identified peripherally in coho salmon (leukocytes & gills), rainbow trout (liver, kidney, muscle, gill, intestine, brain), and brook trout (gills) (Chakraborti *et al.*, 1987; Pottinger, 1991; Lee *et al.*, 1992; Shrimpton & Randall, 1994; Teitsma *et al.*, 1997; Allison & Omeljaniuk, 1998) and characterised in the brain of chinook salmon (*Oncorhynchus tshawytscha*) brain (Knoebl *et al.*, 1996). More recently, high densities of these receptors have been localised throughout the PON and NLT (Teitsma *et al.*, 1997) of rainbow trout brain, with all irCRH neurones in the PON expressing these receptors (Teitsma *et al.*, 1998). Thus, the capacity to respond to steroid feedback exists in fish, as one would expect in order to allow a return to basal activity at all times.

In fish, as in mammals, a constituent of the negative feedback mechanism may be a reduction in the number of cortisol receptors available, thus effectively decreasing sensitivity to the steroid (Allison & Omeljaniuk, 1998). In the rainbow trout, receptor numbers appear to be rapidly depleted following cortisol injection with an inverse relationship between receptor-like binding of cortisol and plasma steroid concentration (Pottinger, 1991). A similar downregulation occurs in the brain following dexamethasone treatment (Lee *et al.*, 1992). In coho salmon, chronic elevation of cortisol, induced by steroid treatment or repeated daily handling, reduces gill cortisol receptor concentration, which persists even after plasma cortisol returns to basal titres (Shrimpton & Randall, 1994).

Cortisol also appears to inhibit peptide hormone release in fish. In the absence of cortisol, CRH, AVT, and ACTH immunostaining is increased as peptide release is upregulated (Olivereau & Olivereau, 1991*b*), while the addition of exogenous cortisol acts in a reverse manner, depressing irACTH in the corticotropes of the eel (Olivereau & Olivereau, 1988*b*). Furthermore, CRH-induced ACTH release from cultured pituitary cells can be inhibited by cortisol (Fryer *et al.*, 1984), and the stress-related release of ACTH prevented by dexamethasone (Pickering *et al.*, 1987). Treatment with exogenous cortisol will also eliminate the rise in plasma cortisol after acute handling (Barton *et al.*, 1987). In the goldfish, CRH mRNA in the telencephalon-preoptic region is reduced following cortisol treatment, and increased following treatment with the glucocorticoid antagonist, RU486 (Bernier *et al.*, 1999). Quantitative studies on the effects of cortisol on parvocellular AVT or CRH transcripts in the fish stress center, the PON, have yet to be carried out.

Aims

Previous work described in this thesis has shown that POMC gene transcript abundance is increased along with parvocellular AVT transcripts following acute stress (see Chapter 4). In this study the aim was to investigate the ability of cortisol to depress gene transcripts in both the PON and pituitary corticotropes and thus gain a further insight into the regulatory mechanisms involved in cortisol negative feedback in the trout.

5.2 Additional Material and Methods

Methodologies specific to this study were employed as described below.

5.2.1 Fish

Rainbow trout husbandry was as described previously (see Section 2.1) for all experiments with the exception of Expt 2, in which fish were obtained from a local trout farm (Alderley Trout Farm, Somerset) and acclimated to the aquarium conditions for four weeks prior to the start of the experiment. All experiments began at approximately 14.00h. At sample times fish were captured by a sweep of a large net.

5.2.2 Cortisol coating of pellets

A monolayer of commercial trout pellets (BOMC Paul Ltd.) was just covered with a 3.4mg cortisol/ml ethanol solution and left undisturbed for 20 minutes. The ethanol was then withdrawn and the pellets allowed to dry at room temperature (typically 12-16 hours). Once treated, pellets were stored in airtight containers at 4°C until use.

A sample of eight to ten treated, dried pellets per batch were assayed to determine the amount of cortisol absorbed by each pellet. Individual pellets were incubated in 2mls of ethanol at 4°C overnight, with the amount of solublised cortisol determined by radioimmunoassay. Cortisol values ranged between 22 - 50μg/pellet after a single coating. This amount could be doubled if pellets were re-treated with the cortisol solution. A variety of soaking times in the cortisol/ethanol solution were tested (between 5 – 20 minutes) though it was found that this did not markedly alter the amount of cortisol per pellet, suggesting that the cortisol coated, rather than penetrated, the pellets.

For control, or 'normal', feed pellets were soaked in a pure ethanol solution for five minutes then treated as described for cortisol treated pellets. This ensured nutritional continuity between cortisol-treated and control pellets since certain ethanol-soluble components of the feed might be leeched out during the treatment process.

5.2.3 Cortisol feeding trials

Cortisol was presented in the feed as a stress-free method of administration. The effect of ingested corticosteroid on plasma cortisol values was tested with a series of preliminary feeding experiments; fish were fed either singly or twice per day at different cortisol doses and the plasma cortisol concentration ascertained by radioimmunoassay at intervals following the feeding (see Figure 5.2). Control fish were kept in adjoining tanks and fed an equal weight of 'normal' pellets. Each treatment group consisted of eight fish.

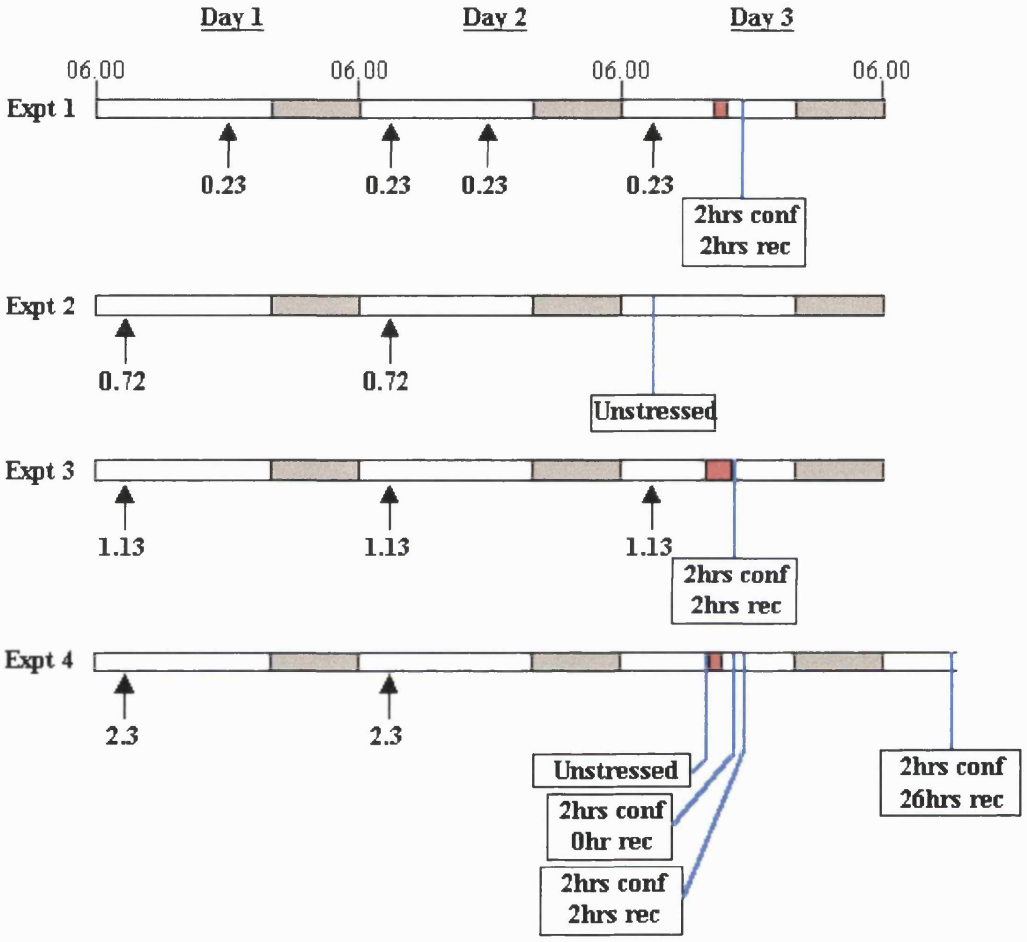
5.2.4 The effect of exogenous cortisol, with or without stress, on AVT and POMC mRNAs

Fish were distributed into two tanks and left undisturbed for at least three weeks prior to the beginning of experiments. Those in one tank received cortisol pellets once or twice a day (see Table 5.1 and Figure 5.1) with controls receiving an equivalent weight of normal pellets. Morning feeds were given at 09.30h, evening feeds at 17.30h. Fish from control and cortisol fed tanks were subjected to confinement stress for two or four hours and killed, following a recovery period in home tank water, between four and 28 hours after the start of the stress. A series of experiments, each using different levels of cortisol feed, were carried out. At the end of each experiment blood was collected for plasma cortisol radioimmunoassay and brains, and pituitaries were fixed for AVT and POMC mRNA determination using *in situ* hybridisation.

Table 5.1; Details of experiments testing the effect of exogenous cortisol, as administered in the food, on AVT and POMC mRNAs. Confinement density is given in parenthesis. n = number of fish per group.

Expt. No.	n	Fish Weight (g±SEM)	Cortisol/meal (mg/kg)	Stress Paradigm	Recovery Period (hrs)
1	8	395±17	0.23	2hrs confined (106g/L)	2
2	8	211±6	0.72	-	-
3	6	308±10	1.13	4hrs confined (191g/L)	0
4	8	143±3	2.3	2hrs confined (141g/L)	0, 2 & 26

Figure 5.1; Cortisol feeding regimes in Experiments 1 to 4. Shading denotes the dark period (lights on from 06.00 – 22.00h). Black arrows show times when cortisol pellets were offered. The value under the arrow shows the amount of cortisol presented (mg cortisol/kg body weight/meal). Red shading denotes when the stressor was applied, with blue lines showing times of autopsy. Unstressed controls were sampled at the same time as stressed fish unless otherwise indicated. conf = confinement, rec = recovery.

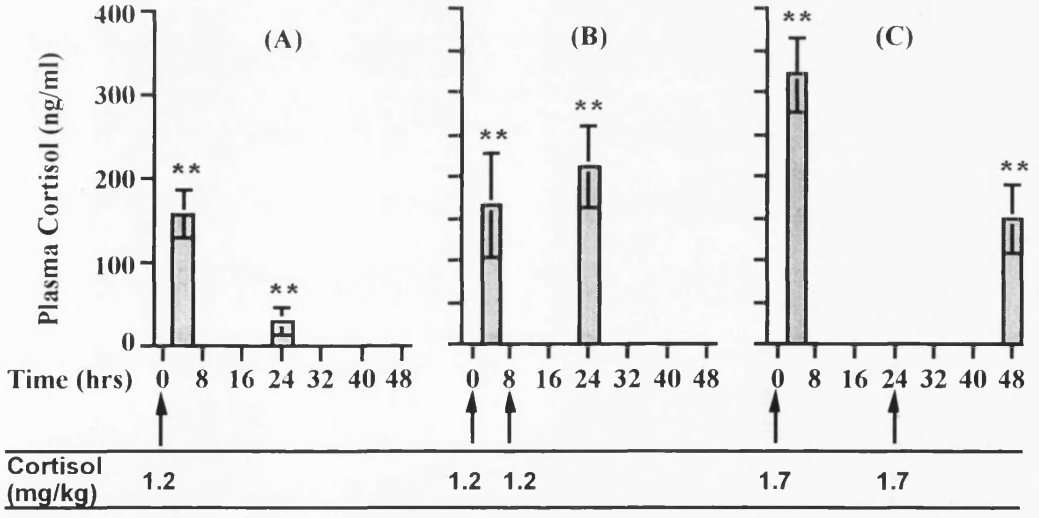


5.3 Results

5.3.1 Effect of feed administered cortisol on circulating levels of the steroid

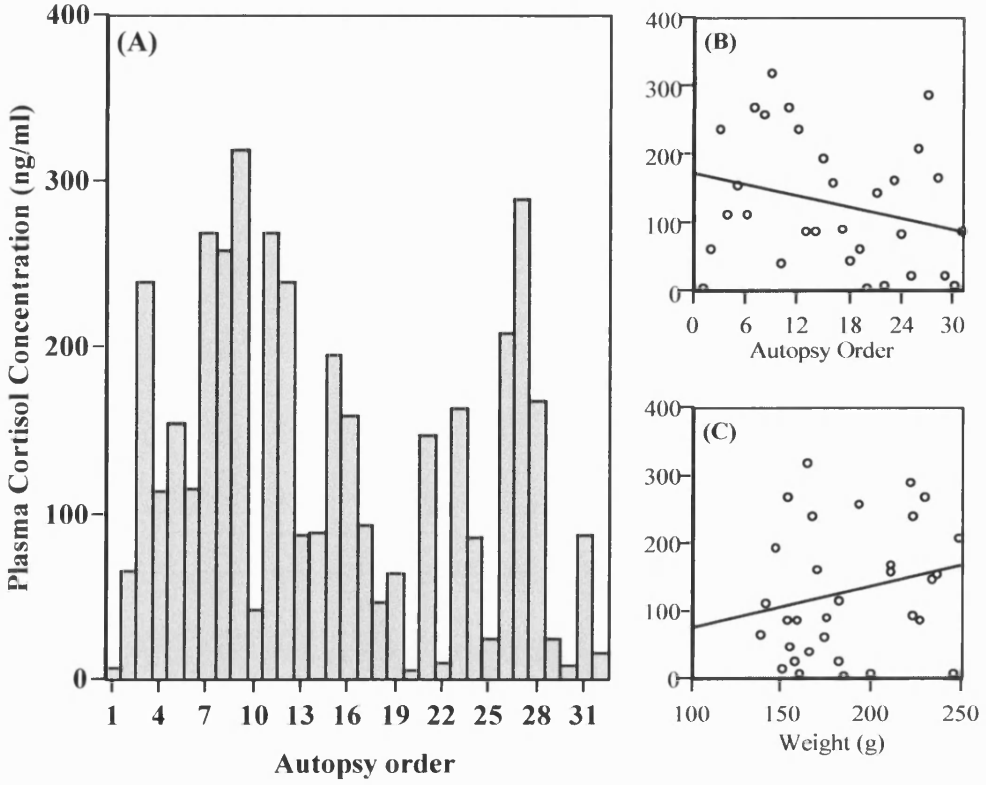
Preliminary trials to determine the change in circulating cortisol concentration associated with feeding steroid-coated pellets found that a single meal significantly raised cortisol titres when measured four hours later (Figure 5.2). Values, although declining, were still high 24 hours after the meal suggesting a continued absorption of cortisol from the gut. The effect of a second meal was to maintain high plasma concentration (Figure 5.2B, C).

Figure 5.2: The effect of ingested cortisol on plasma cortisol titres in unstressed rainbow trout. Bars are means ± SEM for six fish. Fish were initially fed cortisol-coated pellets at 09.00h; all meals are indicated by arrows. Feeding rate per meal was 1.2 (A & B) or 1.7mg cortisol/kg body weight per meal (C). The x-axis is time in hours since the first meal was presented. ** = P<0.01 as compared to control values (one-way ANOVA). Fish fed an equivalent weight of 'normal' pellets had mean cortisol values between 0.4 - 1.5ng/ml (not shown).



Considerable variation occurred in plasma cortisol titres between individual fish; in some cases highest values were as much as ten times the lowest individual value (see Figure 5.3). A regression analysis indicated that this variation was not due to the autopsy order (see Figure 5.3B) or fish size (see Figure 5.3C), though an inspection of stomach contents revealed a difference in the number of pellets consumed which might account for this.

Figure 5.3; Graph showing variability in plasma cortisol titres between fish from the same tank fed two cortisol meals of 0.72mg cortisol/kg body weight/meal. (A) shows the variation in plasma cortisol titres between individuals as compared to autopsy order. Other graphs are regression analyses of, (B) plasma cortisol titre versus autopsy order (r^2=0.08), and (C) plasma cortisol concentration versus fish weight (r^2=0.05).



5.3.2 Effect of feed administered cortisol, with or without stress, on AVT and POMC mRNAs

It is difficult to compare individual experiments because fish were killed at different times following the last meal, but results indicate that the treatment rate appeared to be closely coupled to plasma cortisol titres if one took into account variabilities between feeding and sampling time (see Table 5.2). Results showed that even the lowest level of cortisol treatment, given over two days, depressed POMC mRNA in the pituitary corticotropes in unstressed fish as compared to fish fed 'normal' pellets (see Table 5.2). In contrast, AVT mRNA levels in the preoptic nucleus parvocellular neurones were not

depressed until the highest treatment rate of 2.3mg cortisol/kg body weight (Expt 4). Plasma cortisol concentrations in this experiment (Expt 4) were between 145 and 210ng/ml, however, given the duration between the last cortisol treated meal and the first sampling time, some 28 hours, it is likely that steroid concentrations were elevated well beyond the physiological levels observed at sampling times. The AVT hybridisation signal was significantly depressed at this treatment rate until 54 hours after the last cortisol administration when plasma steroid concentration declined to 42±20ng/ml and AVT mRNA levels returned to near control levels.

Table 5.2; Plasma cortisol values following exogenous cortisol administration, and messenger RNA (mRNA) levels after cortisol treatment in unstressed rainbow trout. Control fish were fed an equivalent weight of 'normal' pellets. *, ** - denotes a significant decline from untreated controls ($P<0.05$ and $P<0.01$ respectively, one-way ANOVA), ns = no significance from controls. Values are means ±SEM. Transcript values are expressed as a percentage of respective controls. nd = not determined.

Expt. No.	Cortisol/ day (mg/kg)	Time since last meal	Plasma cortisol (ng/ml) Cortisol Fed fish	Plasma cortisol (ng/ml) 'Normal' Fed fish	Corticotrope POMC mRNA	AVT mRNA
1	0.46 x2 days	4.5 hrs	59±7	4±1	36±5*	105±14ns
2	0.72 x2 days	24 hrs	10±3	0.4±0.1	11±2**	nd
3	1.13 x3 days	4.5 hrs	>320	4±2	29±12*	139±37ns
4	2.3 x2 days	28 hrs	211±42	2±1	nd	30±9**
		30 hrs	183±41	53±5	nd	30±12**
		32 hrs	145±27	12±2	nd	38±9**
		54 hrs.	42±20	11±4	nd	91±18ns

Unfortunately the stress paradigms used did not elicit changes in either pre-optic parvocellular AVT transcripts or POMC mRNA in the corticotropes of fish fed normal pellets. Consequently, the effect of cortisol on any such changes could not be tested. The possible reasons for this lack of response, by the AVT neurones in particular, have already been covered in Chapter 4.

5.4 Discussion

This work demonstrates that basal POMC transcripts in the corticotropes can be depressed when circulating concentrations of cortisol are raised for 48 hours above 60 ng/ml (Expt 1), the equivalent to that experienced during moderate chronic stress. In contrast, very high circulating cortisol levels, in excess of those likely to be experienced during even severe stress, are required to depress basal AVT mRNA.

A number of different methods of cortisol administration were available, all of which have disadvantages. These include ip injection, use of implants, or presentation of the steroid on the feed. Injection, although allowing for a greater degree of control over the dose dispensed, stimulates the HPI axis (Swift, 1982). The surgery required for implants is prone to this same problem and requires extensive preliminary trials in order to determine dose rates. Presentation of cortisol via the feed has been previously demonstrated to be both stress-free and successful in achieving physiological plasma doses (Pickering & Duston, 1982). A two day treatment period prior to sampling was used in the current study since maximal suppression of the response to stress by the HPI axis in trout (Pickering *et al.*, 1987) and CRH mRNA in the rat (Lightman & Young, 1989) is observed 48 hours following synthetic corticosteroid administration.

In some studies the synthetic corticosteroid, dexamethasone, has been used to study steroidal suppression of the HPI axis (Pickering *et al.*, 1987; Green & Baker, 1991). This allows for confirmation that cortisol secretion has been suppressed since dexamethasone does not cross-react significantly with cortisol antiserum in the radioimmunoassay (Green & Baker, 1991). In the current study the natural form of the steroid, cortisol, was used. This has the advantage of allowing one to determine both the amount of steroid administered and the rise in circulating hormone. Also, it more closely mimics the natural situation since dexamethasone has a slightly higher affinity for the glucocorticoid receptor in salmonids (Allison & Omeljaniuk, 1998; Knoebl *et al.*, 1996).

The observed variability in plasma cortisol titres between steroid-fed fish in the current study could be due to social effects. The social structure of rainbow trout is characterised by the formation of hierarchies (Metcalfe *et al.*, 1989), with the share in the group meal

related to the individual's rank, this generally being determined by fish size (Winberg *et al.*, 1993). Although there was no apparent effect of fish weight on the amount of cortisol consumed such hierarchies, which are largely unavoidable, are likely to have been a contributing factor to variations in the number of pellets eaten.

5.4.1 The effects of corticosteroid negative feedback on the HPI

That the hypothalamic and pituitary hormones implicated in the stress response in fish are, as in mammals, subject to negative feedback by circulating corticosteroids has been known for some time. In fish, normal cortisol titres inhibit the release of CRH, AVT and ACTH as indicated by changes in immunoreactivity and nuclear diameter of perikarya containing these peptides upon the removal of the endogenous steroid (Olivereau & Olivereau, 1988*b*, 1990*b*, 1991*a*, *b*).

A number of other studies have also investigated the ability of high exogenous corticosteroids to inhibit the HPI axis response to stress. In work by Barton *et al.* (1987) 3mg cortisol/kg body weight applied via the food for four weeks raised plasma cortisol titres to 300ng/ml in rainbow trout. This was sufficient to suppress a handling-stress-induced rise in plasma cortisol when compared to untreated controls. Such studies, however, do not discern whether steroid inhibition is occurring at the level of the hypothalamus, pituitary, or both.

In the brown trout the consequence of a single dexamethasone meal on the pituitary–interrenal response to a one hour confinement stress was tested using three dose rates of 1mg, 2mg, and 3mg dexamethasone/kg body weight (Pickering *et al.*, 1987). In all experiments the effects were not seen until 24 hours after the cortisol meal, with any suppressive consequence no longer apparent 96 hours after steroid treatment. The 1mg/kg treatment, although muting the plasma cortisol response as compared to normal fed controls, failed to suppress activation of the HPI axis. At both the 2mg/kg and 3mg/kg dose rates the stress-induced rise in cortisol was restrained. Plasma ACTH in both control and stressed animals was also significantly depressed at the 3mg/kg dosage. This is a comparable treatment rate to that which we find suppresses AVT mRNA in unstressed trout. Taken together it seems likely that both transcription and release of

AVT are being restrained at the highest treatment rate (i.e. 2.3mg cortisol/kg body weight) in the current study.

The effects on ACTH release of 2mg dexamethasone/kg body weight were not tested by Pickering *et al.* (1987). However, this dose acted to constrain the plasma cortisol response to stress. Presumably this is acting on both ACTH release and transcription since in the current work similar cortisol doses (0.7mg/kg x 2days) inhibited POMC gene transcripts. The lowest dexamethasone treatment regime (1mg/kg body weight) failed to restrain the HPI axis, allowing a near normal plasma cortisol response to stress (Pickering *et al.*, 1987). Coupled with the finding in the present study that even the lowest tested doses of cortisol significantly depresses POMC mRNA emphasises that the corticotropes remain able to release ACTH in response to hypothalamic stimulation even when peptide synthesis is restrained by negative feedback.

AVT transcription was not significantly depressed until the highest cortisol treatment level of 2.3mg cortisol/kg body weight (Expt 4). In the goldfish exogenous cortisol treatment using solid silastic implants reduces CRH mRNA in the telencephalon-preoptic region (Bernier *et al.*, 1999) at high doses of 300mg cortisol/kg body weight. This resulted in circulating concentrations of cortisol of 500ng/ml six hours following implantation, declining to approximately 140ng/ml 24 hours after implantation. These plasma values are analogous to those seen in our experiments following the 1.1 and 2.3mg/kg body weight cortisol feeding regimes. It seems, therefore, that pharmacological levels of plasma cortisol, in excess of 450ng/ml, are required to suppress either AVT or CRH mRNAs.

AVT gene transcripts returned to basal levels within two days of the last cortisol meal. This is in contrast to the suppressive effects of 2mg dexamethasone/kg body weight on the cortisol response to stress which persist for three days, and 3mg/kg dexamethasone dose which depresses stress-induced cortisol and ACTH release for the same period (Pickering *et al.*, 1987). This discrepancy is probably attributable to the higher affinity the synthetic steroid has for the cortisol receptor; and the longer half life of dexamethasone which cannot be metabolised (Knoebl *et al.*, 1996; Allison & Omeljaniuk, 1998).

The results of immunocytochemical studies indicate that glucocorticoid receptor-expressing cells are located in a variety of brain regions, including the parvo- and magno-cellular pre-optic region, and in the pituitary pars distalis (Teitsma *et al.*, 1998). In salmonids this single glucocorticoid receptor type has been characterised with a K_D ranging between 1.2 – 4.5nM cortisol (Ducouret *et al.*, 1995; Knoebl *et al.*, 1996; Pottinger & Brierley, 1997; Allison & Omeljaniuk, 1998). This K_D is comparable to plasma cortisol concentrations of 3nM (~1ng/ml) found in unstressed trout. Modest corticosteroid levels may thus be enough to suppress the stress-induced rise in AVT, though under basal conditions gene expression is already constrained by cortisol and suppression below this requires pharmacological doses.

5.4.2 Mammalian comparisons

A comparison of these findings to work done in mammals reveals interesting similarities. In rats, corticosteroids exert a negative feedback effect on the HPA system over three different time periods; less than 15 minutes ('rapid'), within two hours ('early delayed'), and between two and 24 hours ('late delayed'). There is evidence to suggest that the parvocellular CRH and AVP mRNA suppression by circulating glucocorticoids can be very slow (Lightman & Young, 1989; Swanson & Simmons, 1989) and requires sustained high levels of steroid. For example, a dexamethasone dose of 2mg/kg depressed basal and stress-induced CRH when given in five aliquots over a two day period but had no effect when given in a single dose four to six hours prior to autopsy (Lightman & Young, 1989). Ma and co-workers (1997*c*) showed that adrenalectomy-raised levels of AVP mRNA are unaffected four hours after an injection of 10mg/kg corticosterone. Interestingly, AVP heteronuclear RNA (hnRNA), which provides a closer indication of gene transcription changes than mRNA (Ma *et al.*, 1997*c*), fell markedly within 15 minutes of steroid injection remaining depressed up to four hours later. CRH hnRNA was reduced both two and four hours following corticosterone administration, with CRH mRNA also significantly reduced at the four hour time point. Thus, although transcription rate is affected relatively quickly, as adjudged by reductions in hnRNA, effects on the more stable mRNA pool occur at a much slower rate. Intronic sequences for fish AVT and POMC mRNAs have not been determined, consequently it is not currently possible to carry out such studies in teleosts.

5.4.3 Summary

It is apparent that CRH and AVT gene transcripts are modulated by circulating corticosteroids though pharmacological doses of either the synthetic or natural steroid, or adrenalectomy, are required to show this. Thus, although transcripts changes may respond more quickly in response to stress, physiological steroid concentrations may elicit a slower or more modest change that may not be detectable given the stability of large mRNA pools. Furthermore, given this, those treatment time courses used in this study at physiological levels may be of insufficient duration to observe transcripts changes.

Chapter 6

MCH mRNA Responses to Stress and the Possible HPI Modulatory Role of MCH

6.1 Introduction

In teleosts, MCH is involved in physiological colour change, making the fish appear lighter when placed in a pale environment (Rance & Baker, 1979; Baker, 1988, 1991). Circulating MCH acts peripherally to cause melanin aggregation in the melanophores of the skin leading to pallor in direct antagonism to the darkening effects of α-MSH (Baker, 1988; Baker, 1993). Interaction between α-MSH and MCH also occurs within the pituitary with MCH depressing the release of α-MSH from the pars intermedia (Baker *et al.*, 1986; Barber *et al.*, 1987). Plasma concentrations and hypothalamic mRNA levels of MCH are thus higher in fish kept on a white background than those on a black background (Kishida *et al.*, 1989; Suzuki *et al.*, 1995). Indeed, when fish are moved onto a lighter background, hypothalamic and pituitary MCH peptide stores are depleted as plasma concentration increases (Rance & Baker, 1979; Barber *et al.*, 1987; Powell & Baker, 1987).

Further to its role as a skin pallor hormone, MCH also appears to influence the HPI axis response to stress, with fish kept in pale coloured tanks displaying lower ACTH and cortisol concentrations following moderate stress than those kept on a black background (Baker & Rance, 1981). Evidence suggests that this influence is not exerted at the interrenal (Baker *et al.*, 1985*b*) but at both pituitary and hypothalamic level. MCH administered either *in vitro* or *in vivo* depresses the release of ACTH from the pituitary gland (Baker *et al.*, 1985*a*, 1986), with both ACTH and cortisol secretion reduced when plasma MCH is high (Green & Baker, 1991; Gilham & Baker, 1985). Furthermore, MCH acts to depress the *in vitro* release of one or other of the corticotropin secretogogues (Green *et al.*, 1991). This inhibitory effect is likely to be enhanced under certain conditions of stress since MCH release is upregulated in such instances (Green & Baker, 1991; Green *et al.*, 1991). Whether MCH acts to reduce only CRH/AVT release or also influences CRH/AVT synthesis is not known.

Aims

The work described in this chapter examines several aspects of MCH's involvement with stress. First (published paper) it investigates the response of the two major groups of

MCH neurones – NLT (nucleus lateralis tuberis) and LVR (lateral ventricular recess) – to the acute and chronic stresses which were shown in Chapter 4 to either stimulate or depress AVT synthesis in these same fish. Secondly, given the ability of MCH to depress ACTH release, the relative levels of AVT transcripts in trout reared in either white or black tanks and subjected to stress was studied. This difference in tank colour is known to markedly affect MCH secretion; it was of interest to determine whether high MCH secretion depressed AVT and POMC transcripts, or whether MCH depresses only by an effect on AVT and/or ACTH release.

Finally, experiments were performed to see whether the difference in cortisol secretion between fish reared in white or black tanks was apparent only after mild stress, or whether it could be exhibited also in more intensely stressed animals.

6.2 Published Paper

The Influence of Acute or Chronic Stress of Different Duration on MCH mRNA Abundance in Rainbow Trout (*Oncorhynchus mykiss*)

Gilchriest, B. J., Tipping, D. R., Levy, A., and Baker, B. I.
Recent Developments in Comparative Endocrinology and Neurobiology, 1999, p 135-139.
(Roubos, E.W., Wendelaar-Bonga, S.E., Vaudry, H., & Loof, J.G., eds.)

In this paper the majority of the work described was carried out by B.Gilchriest. Dr. D.Tipping assisted with the end-labelling and selection of probes. Facilities for labelling oligonucleotide probes were provided for by A.Levy. Dr. B.I.Baker supervised the work.

The Influence of Acute or Chronic Stress of Different Durations on MCH mRNA Abundance in Rainbow Trout (*Oncorhynchus mykiss*)

B.J. Gilchriest[1], D.R. Tipping[1], A. Levy[2] & B.I. Baker[1]

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Abstract - Adult rainbow trout (*Oncorhynchus mykiss*), reared in pale-colored tanks to free their MCH neurones from inhibitory background colour control, were subjected to either a brief (2 mins low water) or a more prolonged stress (2 hr confinement). Fish were given either a single stress only, or it was repeated daily for 5 or 6 days. Four hours after the start of the stress period, the fish were killed and the abundance of MCH mRNA expressed in the two major groups of MCH neurones was determined by quantitative *in situ* hybridization. In contrast to several earlier findings, stress depressed MCH mRNA abundance but only after repeated application and only in the magnocellular MCH neurones. Neuroregulatory factors that might influence MCH gene expression are briefly discussed. The results suggest that if the type of stress determines the direction of transcriptional response, this might account for the reported variation in response between fish and rats.

INTRODUCTION

The melanin-concentrating hormone, MCH, is believed to have several functions in teleost fish. In addition to its undisputed role in skin pigmentary control, it also seems to modulate the animal's response to stress. Several studies on trout show that when endogenous MCH release is high, the ACTH and cortisol response to stress is reduced (Gilham & Baker, 1985; Green & Baker, 1991). Exogenous MCH in both trout and rats also restrains ACTH release (Baker *et al.*, 1986; BluetPajot *et al.*, 1995), an effect probably exerted largely at the hypothalamic level (Green *et al.*, 1991).

Conversely, the effects of stress on MCH synthesis and release are less clear and paradoxically, the literature suggest that the MCH synthesis and release is enhanced by stress, while in rats it is diminished. In teleosts the dominant factor regulating MCH neuronal activity is background colour and the effects of stress on MCH synthesis and release are clear only in fish kept in a pale environment, when MCH neurones are dis-inhibited. In trout kept under such conditions, a brief daily-repeated stress, such as an injection of isotonic saline without anesthesia, or one minute immersion in ice-cold water, have been found to enhance MCH release (Green and Baker, 1991), MCH synthesis (Baker & Bird, 1992) or MCH mRNA abundance (Suzuki *et al.*, 1997) in the magnocellular MCH neurones of the nucleus lateralis tuberis (NLT). A second cluster of

MCH neurones associated with the lateral ventricular recess (LVR) showed similar increases in MCH mRNA levels after a daily cold shock (Suzuki *et al.*, 1997) or following mild daily disturbances in another teleost, *Oreochromis mossambicus* (Gröneveld *et al.*, 1995). On the other hand, twice-daily stresses or continuous osmotic challenge have been found to depress MCH synthesis or mRNA levels, both in trout (Baker & Bird, 1992; Francis *et al.* 1997) and in rats (Presse *et al.*, 1992).

The aim of the present study was to investigate the effect on MCH mRNA abundance, of two durations of stress (2 min or 2 hrs) applied either once or on a daily basis for 5 or 6 days.

MATERIALS & METHODS

Fish. Rainbow trout (*Oncorhynchus mykiss*) were reared in our aquarium in white tanks (11°C; 16:8 light:dark cycle) and used for these experiments at 16 months.

Administration of stress. Control fish were undisturbed throughout the experiment. Stressed fish were subjected to either a confinement in 15 l of aerated, flowing water for 2 hrs (Expt. A - 214 g/l; Expt. B - 56.5 g/l), or two minutes in low water (Expt. C - 2" depth). All experiments started at 14.00 h and were terminated at 18.00 h when controls were also killed. The stress was applied either once or was repeated daily for five (Expt. A) or six (Expt. B) days.

Collection and preparation of tissues. Fish were anaesthetized in water containing 0.06 % phenoxyethanol (Sigma Chemical, Poole, Dorset, UK) and blood collected from the severed caudal vessels for cortisol measurement. The brain was fixed in 4 % paraformaldehyde in 0.05 M phosphate-buffered saline, pH 7.6. Following wax embedding, serial sections cut at 10 µm were mounted on gelatinized slides for *in situ* hybridization.

In situ hybridization. The antisense oligodeoxynucleotide probe (Perkin-Elmer, Warrington, Cheshire, UK) was directed against the mRNA coding for the mature MCH: TCC CAC CAT GCA CCT CAT GGT GTC. The probe, labeled at the 3' end with [α-^{35}S]dATP, was applied in hybridization buffer at 3.10^5 cpm/10µl. Hybridization proceeded overnight at 37 °C, and excess label was removed by washing at 55 °C in 1xSSC. After exposure to autoradiographic film (Hyperfilm MP, Amersham, UK) the area and density of the signal was monitored by computer densitometry. For further details see Suzuki *et al.* (1997).

Plasma Cortisol Radioimmunoassay. Plasma cortisol concentration was measured by radioimmunoassay (Rance & Baker, 1981).

RESULTS & DISCUSSION

When stress was applied once only and the fish killed 4 hrs after the start of the stress, MCH mRNA values in both the magnocellular NLT and the parvocellular LVR neurones were similar to those of control fish, whether the stress lasted for 2 min or 2 hrs (Fig. 1a). Plasma cortisol values were still significantly above basal at the time of autopsy despite 4

or 2 hrs recovery after stress. This lack of response is in keeping with the finding that a single stress is ineffective in raising plasma MCH titers, although daily repetition of the stress is stimulatory (Green & Baker, 1991). In the present experiments, in contrast to some previous studies, daily-repeated stress significantly depressed MCH mRNA in the NLT-MCH neurones in two out of the three experiments, whether the stress was of brief or prolonged duration (Fig. 1b). The LVR neurones again showed no significant response.

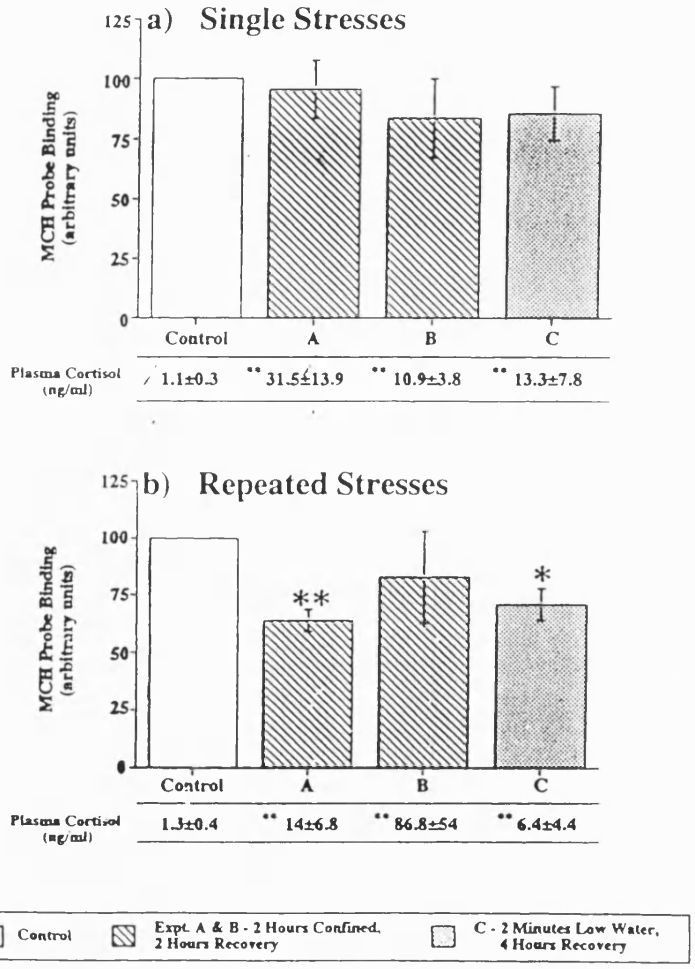


Fig. 1. The effect of stress on MCH messenger RNA in the nucleus lateralis tuberis (NLT). Fish were stressed either singly (a), or repeatedly (b) for five or six days. * P<0.05, ** P<0.01, one-way ANOVA, compared with controls.

It is not clear why stress will sometimes stimulate and at other times depress MCH gene expression. In previous studies in which MCH release or MCH gene expression were enhanced, the repeated stress was of short duration whereas continuous osmotic stress was initially stimulatory and then depressive (see Introduction). One purpose of the present work was to determine whether stresses of different durations had opposite effects. This did not seem to be the case - both transfer to low water for 2 min or 2 hrs confinement depressed MCH mRNA levels in the NLT when repeated daily for 5 days. Further studies will be needed to determine if the physical or psychological component of stress is important.

At present, therefore, we are unable to predict the way MCH gene transcription will be affected by stress. The activity of MCH neurones is most likely controlled by several inputs. Higher brain centers may transmit the perception of stress. Glucocorticoids influence stress-induced MCH release (Green and Baker, 1991) and MCH transcription (Presse *et al.*, 1992); since the NLT-MCH neurones possess glucocorticoid receptors (Kah, personal communication) the effects of cortisol could be direct as well as indirect. In addition, the corticotrophic releasing factor, CRF, which is released abundantly during stress, can influence MCH synthesis and release in rat hypothalamic cultures (Parkes & Vale, 1993) and thus might modulate MCH secretion also in fish. Immunoreactive CRH cell bodies or fibers have been described near both groups of MCH neurones in the trout (Olivereau & Olivereau, 1988). Interplay between these factors, leading to changes in their relative emphasis, may account for the variable response of the MCH neurones. Such interplay, or dynamic changes in the responsiveness of the MCH neurones to one or other regulator, could explain the observation in both the trout (Francis *et al.*, 1997) and in the rat (Presse *et al.*, 1992) that the changes in MCH mRNA may be transitory, and return to basal within a week even when stress persists.

That a secretory cell can display contradictory responses to a particular stress is by no means unknown in endocrinology. Thus, the secretion of α-MSH by the trout pituitary pars intermedia may be enhanced (Sumpter *et al.*, 1986) or depressed (Balm & Pottinger, 1995) by confinement stress implying, as for MCH, the involvement of multiple control pathways.

These findings suggest that the difference that was initially proposed between rat and trout MCH gene transcriptional response to stress (Baker, 1994) may be attributable more to the intensity or nature of the stress employed rather than reflecting a real difference between fish and mammals in MCH control.

Acknowledgements - We thank the BBSRC for financial support.

REFERENCES

Baker BI (1994) Melanin-concentrating hormone updated. Functional considerations. Trends Endocrinol. Metal. 5: 120-126

Baker BI & Bird DJ (1992) The biosynthesis of melanin-concentrating hormone in trout kept under different conditions of background colour and stress, as determined by an *in vitro* method. J.

Neuroendocrinol. 4: 673-679

Baker BI, Bird DJ & Buckingham JC (1986) Effects of chronic administration of melanin-concentration hormone on corticotrophin, melanotrophin and pigmentation in the trout. Gen. Comp. Endocrinol. 63: 62-69

Balm PHM & Pottinger TG (1995) Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 98: 279-288

BluetPajot MT, Presse F, Voko Z, Hoeger C, Mounier F, Epelbaum J & Nahon J-L (1995) Neuropeptide-E-I antagonizes the action of melanin-concentrating hormone on stress-induced release of adrenocorticotropin in the rat. J. Neuroendocrinol. 7: 297-303

Francis K, Suzuki M, & Baker B (1997) Responses of melanin-concentrating hormone mRNA to salt water challenge in the rainbow trout. Neuroendocrinology 66: 195-202

Gilham I & Baker B (1985) A black background facilitates the response to stress in teleosts. J. Endocrinol. 106: 99-105

Green JA & Baker BI (1991) The influence of repeated stress on the release of melanin-concentrating hormone in the rainbow trout. J. Endocrinol. 128: 261-266

Green JA, Baker BI, & Kawauchi H (1991) The effect of rearing rainbow trout on black and white backgrounds on their secretion of melanin-concentrating hormone and their sensitivity to stress. J. Endocrinol. 128: 267-274

Gröneveld D, Balm P, Martens G & Wendelaar Bonga S (1995) Differential melanin-concentrating hormone gene expression in two hypothalamic nuclei of the teleost tilapia in response to environmental changes. J. Neuroendocrinol. 7: 527-533

Olivereau M & Olivereau J (1988) Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. Peptides 9: 13-21

Parkes D & Vale W (1993) Secretion of melanin-concentrating hormone and neuropeptide-E-I from cultured rat hypothalamic cells. Endocrinology 131: 1826-1831

Presse F, Hervieu G, Imaki T, Sawchenko PE, Vale W & Nahon J-L (1992) Rat melanin-concentrating hormone messenger ribonucleic acid expression: marked changes during development and after stress and glucocorticoid stimuli. Endocrinology 131: 1241-1250

Sumpter JP, Dye HM & Benfey, TJ (1986) The effects of stress on plasma ACTH, α-MSH, and cortisol levels in salmonid fishes. Gen. Comp. Endocrinol. 62: 377-385

Suzuki M, Bennett P, Levy A & Baker BI (1997) Expression of pro-MCH and POMC genes in rainbow trout (*Oncorhynchus mykiss*) during ontogeny and in response to early physiological changes. Gen. Comp. Endocrinol. 107: 341-350

6.3 Additional Materials and Methods

Experiments in addition to those detailed in the published paper were carried out as described below.

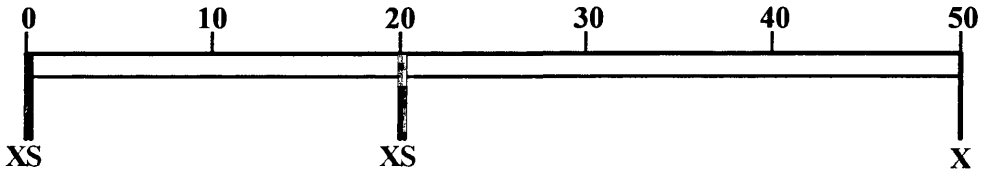
6.3.1 Expt 1; the effect of black or white backgrounds on basal AVT mRNA

This was designed to observe basal AVT transcripts in fish raised from eyed eggs on either a black or a white background. A sample of eight fish (mean weight, 307±7g) were quickly removed from adjoining black and white tanks and anaesthetised in 0.06% phenoxyethanol (Sigma). Following collection of blood for cortisol radioimmunoassay, brains were removed for AVT *in situ* hybridisation.

6.3.2 Expt 2; the effect of mild acute stress and background colour on cortisol secretion

Control fish from neighbouring black and white tanks were removed at 13.00h, anaesthetised and blood was collected for cortisol radioimmunoassay. Fish from these two tanks were then alternately stressed for ten second periods by chasing with a net until each tank had been treated for a total of one minute. This was applied twice as shown in Figure 6.1. Each group consisted of eight fish of mean weight 172±6g.

Figure 6.1; Stress and sampling regime for Expt 2. Red lines labelled 'S' indicate times of stress, with blue lines labelled 'X' denoting times of autopsy. The numerical axis shows the time in minutes since starting the experiment.



6.3.3 Expt 3; the effect of severe acute stress and background colour on cortisol secretion and AVT, POMC and MCH transcripts

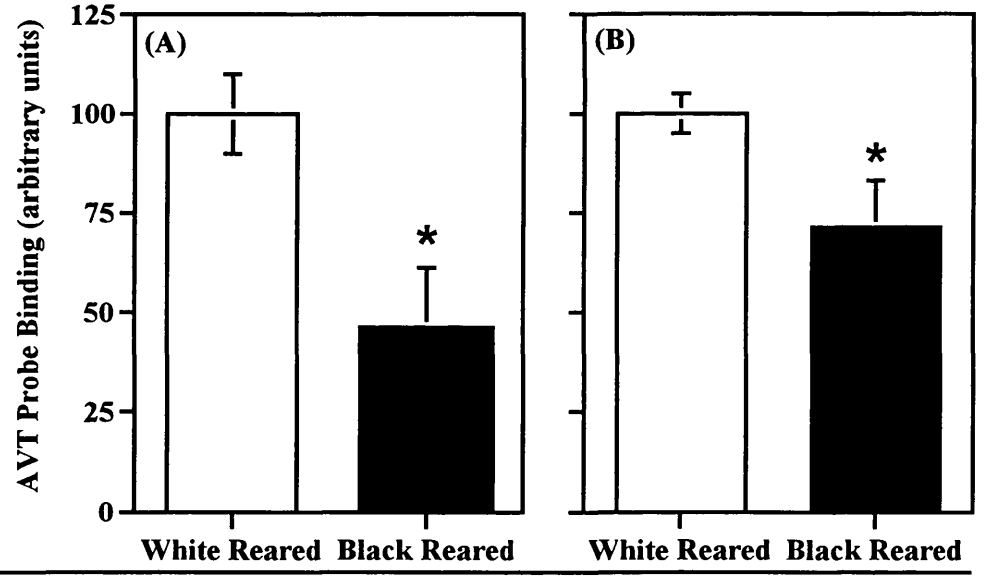
Control fish from a black and adjacent white tank were killed at 12.30h. Transcript data from these control fish was also used to confirm the effect of long term rearing on different tank colours on basal AVT mRNA levels (as Expt 1). At the same time sixteen fish from each background colour were transferred to a small tank (30 x 40 cms), of the same colour as the home tank, containing water to a depth of 4cm such that fish were unable to remain upright (i.e. low water stress). These fish were disturbed by netting and moving the tank for one minute periods every ten minutes, beginning at the time of transfer, over the course of an hour. Animals were sampled immediately following the stress or following four hours of recovery in their home tank. Following anaesthetisation, blood was collected for cortisol radioimmunoassay, and brains and pituitaries were removed for AVT, MCH, and POMC *in situ* hybridisation; POMC transcripts in the melanotropes and MCH mRNA levels in the LVR were not determined. There were eight fish in each group, with a mean weight of 108±4g.

6.4 Additional Results

6.4.1 Expt 1; the effects of black or white background on basal AVT mRNA

Values of basal AVT mRNA from both Expt 1 and control animals from Expt 3 are shown in Figure 6.2. The AVT transcript values were significantly higher ($P<0.05$, one-way ANOVA) in white reared than black reared fish in both cases, though the difference was not as marked in control animals from Expt 3. Plasma cortisol values were <1ng/ml in all fish, with no difference in basal plasma cortisol titres between fish raised on different background colours.

Figure 6.2; Basal AVT transcript levels and plasma cortisol titres of fish reared from eyed-eggs on a white (open bars) or black (closed bars) background. Results shown are from Expt 1 (A) and control animals from Expt 3 (B). Bars are means ± SEM. * = statistical difference from controls ($P<0.05$ as determined by one-way ANOVA).

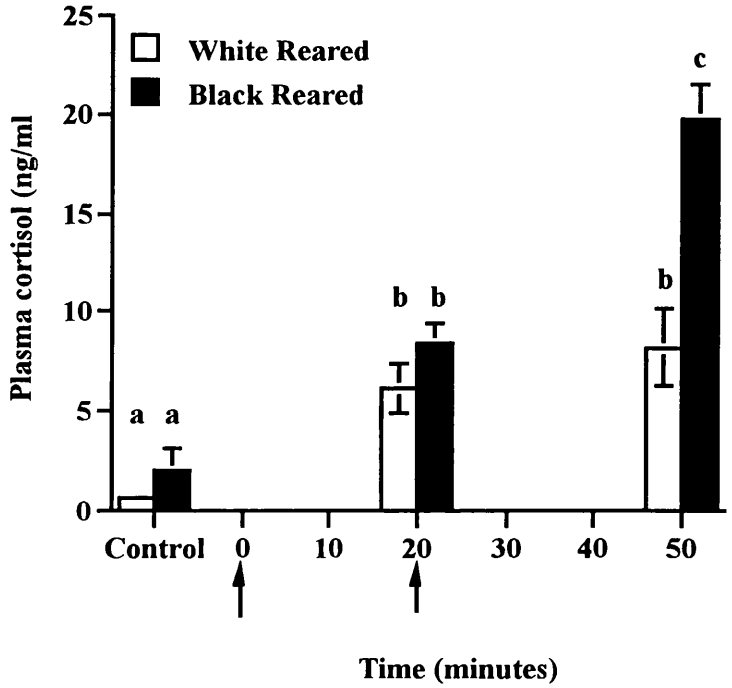


Plasma Cortisol (ng/ml)	0.5±0.1	0.4±0.1	0.4±0.2	0.4±0.1

6.4.2 Expt 2; the effects of mild acute stress and background colour on cortisol secretion

Results shown in Figure 6.3 are in agreement with previous findings (Baker & Rance, 1981; Gilham & Baker, 1985) in that the cortisol response to a mild stress is greater on a black background. Plasma cortisol concentrations in control fish were identical at ~1ng/ml for both tank colours. Twenty minutes following the initial stress plasma cortisol titres were significantly raised from controls though no background effect was evident (white=6±1ng/ml; black=8±1 ng/ml). An additional stress failed to raise plasma cortisol concentration further in white reared fish, while titres in black reared fish more than doubled to 20±2ng/ml.

Figure 6.3; Plasma cortisol response to mild chasing stress in fish reared on a white (open bars) or black (closed bars) background (Expt 2). Fish were stressed for one minute by being pursued by a net at times indicated by arrows. Bars are means ± SEM. Superscripts were assigned following two-way ANOVA ($P<0.05$); bars with different superscripts are significantly different from one another.



6.4.3 Expt 3; the effect of severe acute stress and background colour on cortisol secretion and AVT, POMC and MCH transcripts

Results are shown in Figure 6.4.

Plasma Cortisol

Basal plasma cortisol titres were less than 1ng/ml in fish from both tank colours. The severe, one-hour stress significantly raised cortisol concentrations above those of controls, however, in contrast with previous findings, titres in white reared fish were similar to those in black reared animals. Following four hours recovery in home tanks, plasma cortisol declined significantly though they had not returned to basal levels.

Parvocellular AVT mRNA

As previously described (see Figure 6.2B), AVT mRNA in white reared controls exceeded that of black reared animals. White reared animals showed little change from basal levels following stress. Despite a post-stress increase of 38% in AVT transcript levels (see Figure 6.4) in black reared fish this rise was not significant.

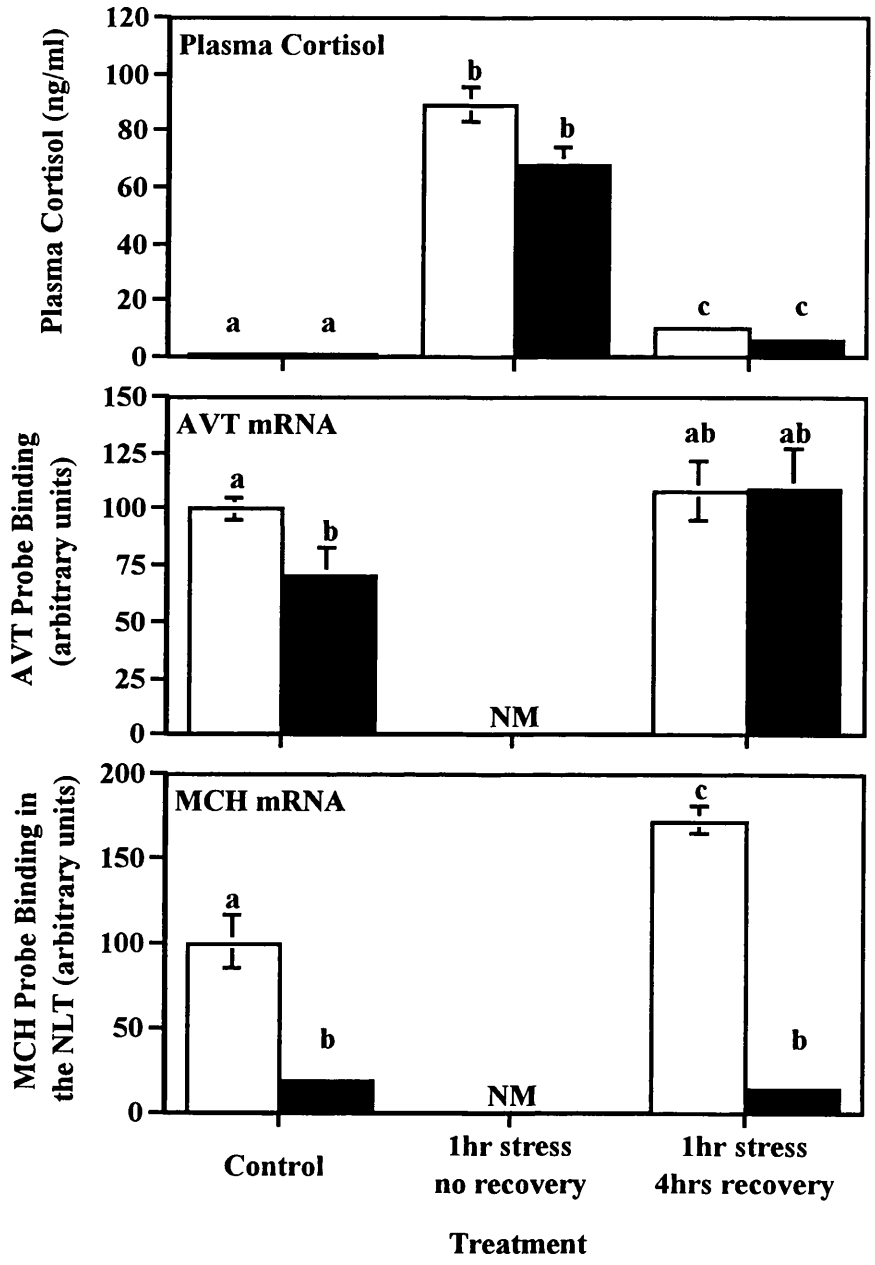
Pituitary POMC mRNAs

There were no significant effects of background on corticotrope POMC transcripts in control fish or following stress.

MCH mRNA

In the principal MCH nucleus, the NLT, MCH messenger RNA was over fivefold higher in white reared than black reared counterparts as expected. Although the stress had no effect on MCH probe binding in black reared animals, in white reared fish transcripts were significantly raised above those of control animals.

Figure 6.4: Results of Expt 3: trout were given a one hour low water stress with periodic disturbance. Fish were sampled immediately following the stress or following four hours recovery. The histograms show relative changes in plasma cortisol concentration, parvocellular AVT probe binding, POMC probe binding in the pituitary corticotropes, or MCH probe binding in the nucleus lateralis tuberalis (NLT). Open bars denote white reared fish; closed bars, black reared fish. Bars are means ± SEM. Superscripts were assigned following two-way ANOVA ($P<0.05$). NM = not measured.



6.5 Additional Discussion

The main points already covered in the published paper (Section 6.2) are summarised below in order to put the additional discussion into context. The results suggest that the duration of stress does not necessarily determine the directional response of MCH transcripts. Thus, either a two minute low water, or two hour confinement stress result in a uniform lack of MCH mRNA response in either the NLT or LVR. When these stressors are applied repeatedly for five days, however, MCH transcripts in the NLT are downregulated; transcripts in the LVR remain unaffected. When administered daily for six days the two hour confinement fails to significantly reduce either NLT or LVR MCH mRNA. These findings are in contrast to prior work on MCH release (Green & Baker, 1991), hypothalamic content (Green *et al.*, 1991), and transcription (Suzuki *et al.*, 1996, 1997) which find that a single stress has no effect, whilst the same stresses applied repeatedly upregulate MCH. The differences in MCH neurone responses between studies is likely to be due to interplay between the multiple controls that are believed to influence MCH, including glucocorticoid negative feedback (Green & Baker, 1991; Presse *et al.*, 1992), and the possible influence of corticotropic releasing factor (Parkes & Vale, 1993).

6.5.1 The effect of background colour on the plasma cortisol response to stress

The background colour-dependant difference in the plasma cortisol response to stress has been well documented. Following noise stress (Gilham & Baker, 1985) or periodic transfer to a new tank (Baker & Rance, 1981) the plasma cortisol titres in black-reared fish were higher than those in white reared animals in which endogenous MCH is higher. However, results here show that this modulatory effect of MCH over the HPI axis only acts when the stress is mild. This is in agreement with other workers (Pickering *et al.*, 1986) who failed to observe an effect of background colour on the stress response following 30 minutes of confinement or a thermal shock. Thus, a more severe stress, resulting in much higher plasma cortisol levels, removes the background-specific difference in the cortisol response to stress. This seems likely to be a result of the intensity of the stressor. Indeed, although the cortisol response in black reared animals remains significantly elevated above that of white reared fish following noise stress, the comparative difference is reduced as stress intensity is increased (Gilham & Baker,

1985). Presumably if the stressor reached sufficient intensity the difference in plasma cortisol response between backgrounds would be lost.

6.5.2 The effect of background rearing colour on basal AVT transcripts

The tendency for lower levels of ACTH release in white- over black- reared trout is thought to be due to a depressive effect of MCH on PON neuropeptides. Given this one might expect reduced levels of ACTH-secretogogue transcripts, such as AVT, in the hypothalamus of white reared trout. However, current results have shown the inverse to be the case with significantly *higher* AVT mRNA levels in white-reared trout. A similar observation has been previously reported; Green and co-workers (1991) found that the hypothalami of white-reared trout release more corticotropin-releasing factor bioactivity than black-reared trout when incubated *in vitro*. It is possible that this may be due to a tendency for cortisol levels to be higher following mild stress in black-reared fish. Although basal cortisol titres are similar, regardless of tank colour, periods of mild, intermittent stress, such as occur in the course of normal aquarium procedures, would result in intermittent periods of greater cortisol secretion in black-coloured tanks. This would lead to an increased cortisol negative feedback effect in black-reared trout and thus reduce AVT transcripts. That cortisol can exert a restraining influence over basal and stress-induced CRH and AVP transcripts in the PVN is well established in the rat (Lightman & Young, 1989; Lightman & Harbuz, 1993). In trout glucocorticoid receptors are expressed on both CRH (Teitsma *et al.*, 1998) and AVT (Bond *et al.*, 2000) parvocellular neurones of the PON, and there is some evidence to suggest cortisol exerts a restraining influence on CRH and AVT secretion (Olivereau & Olivereau, 1990*b*, 1991*a*, *b*). The higher AVT content of fish kept on a white background could therefore be the result of both a higher synthesis and to MCH-restraint on release.

Alternatively, exposure to the white background could in itself be a stressor with the increased AVT gene transcripts reflecting this. Whatever the regulatory mechanism it appears that raising fish on a white background enhances AVT synthesis which could potentially be detrimental. Although MCH exerts a modulatory effect that acts to depress ACTH release, this effect is only evident following milder stresses. Indeed, the increased stores of AVT in white reared animals could result in an elevated response in white- over black- reared animals if a stressor was of sufficient severity. The modulatory effect of

MCH over the HPI is evidently complex and operates within specific constraints. Further work on different stress types, duration, and variations in the number of repetitions would help to clarify our understanding of this modulatory effect.

6.5.3 NLT MCH and the response to acute or chronic stress

MCH transcripts in the NLT are unaffected by either acute low water (2 mins) or confinement (2 hrs). This is in agreement with previous studies in which MCH mRNA remains unchanged following five minutes of low water (Suzuki *et al.*, 1996). However, when low water is applied for one hour and combined with disturbance, MCH transcripts in the NLT increased. Presumably the disparity in the response to acute stress is a function of stress severity, though additional experiments would be required to determine if such a correlation exists.

In the case of chronic stress, increased MCH translation occurs in response to a once-daily injection stress (Baker & Bird, 1992) though translation is reduced if a further stressor, three minutes of low water, is applied six hours later. This could be due to the increased cortisol negative feedback effect that results from prolonged raised cortisol titres. Suzuki *et al.* (1997) found enhanced transcript levels of MCH in response to a repeated, mild (1 min cold water) stress in trout fry, and to three low water stressors (2 mins each) applied on the same day in adults (Suzuki *et al.*, 1996). Francis and co-workers (1997) observed elevated MCH mRNA following prolonged exposure to osmotic challenge (80% seawater), which caused only a slight cortisol increase. However, transcripts were reduced when the osmotic challenge (100% SW) and cortisol rise were more severe. In the current study, somewhat surprisingly, both mild and more intense chronic stress reduced MCH mRNA in the NLT. At present it is still not possible to anticipate how MCH will respond to stress. The mechanism following chronic stress appears to be complex and is likely to be due to differences in the stress severity, cortisol negative feedback on the MCH neurones, or a possible habituation to certain stressors.

6.5.4 LVR MCH mRNA responses to stress; comparison with NLT responses

Transcript responses in the LVR are, generally, in contrast to those seen in the NLT. Thus, following repeated two hour confinement or two minutes low water, MCH mRNA

was downregulated in the NLT while remaining unchanged in the LVR. Although both groups respond similarly to environmental colour (Suzuki *et al.*, 1996) their divergent response to some forms of stress is not unique to this study. Thus, in rainbow trout, chronic low water stress enhances MCH gene transcription in the NLT, but not LVR neurones (Suzuki *et al.*, 1996), while chronic disturbance upregulates pre-proMCH mRNA in the LVR, but not the NLT of the tilapia (Groneveld *et al.*, 1995).

6.5.5 The pituitary POMC response in the corticotropes to stress and background colour

There was no clear difference in POMC levels in the corticotropes under basal or stress conditions. If plasma cortisol titres are lower on white, as previously mentioned (Section 6.5.2), then one might expect higher POMC mRNA levels in fish reared on a light background as a result of the reduced cortisol negative feedback effect. Conversely, the reduced CRF-like bioactivity and ACTH released on a white background might lead one to expect lower corticotrope POMC transcripts. Recent studies show that AVT cannot enhance corticotrope POMC transcription *in vitro* though forskolin, which mimics the effects of CRH (Nakai *et al.*, 1991), will do so (personal communication; Dr.Tipping). Given that POMC mRNA transcription is probably induced by CRH rather than AVT it is difficult to interpret changes to POMC mRNA without knowing how CRH transcripts are affected by MCH.

6.5.6 Summary

These results question the validity of raising fish on a white background in order to modulate the stress response in an aquaculture environment. The differences in response to stress of different severity, particularly that of cortisol, that occur between fish adapted to different background colours demonstrates this. Given the apparent adjustments in the HPI with respect to AVT stores when fish are raised on a white background, it appears that long term rearing in white tanks may be detrimental under certain conditions of stress; the increased endogenous MCH that occurs resulting in elevated AVT, and possible other CRF, transcript levels.

Chapter 7

General Discussion

To put the General Discussion into context the key findings of this work are summarised below;

Key Findings, Chapter 3 - Diurnal Expression of AVT and POMC Transcripts

- AVT mRNA in the parvocellular neurones displayed a clear dirunal rhythm with values highest during the light period.
- The dirunal variation in plasma cortisol concentration was almost the inverse of that of AVT mRNA in the parvocellular neurones, with values lowest during the day and highest during the dark period.
- AVT peptide concentration in the hypothalamus and pituitary showed no significant variation over the 24 hour period.
- There was no variation in the AVT mRNA values in the magnocellular neurones over the 24 hour study period.
- In the pars intermedia the POMC genes, A and B, displayed inverse patterns of transcription suggesting differential regulation. POMC-A transcripts were highest in the morning, declining to a night-time nadir, whilst POMC-B mRNA progressively increased through the day and peaked during the dark period, suggesting differentially regulation.
- There was no diurnal change in POMC-A mRNA in the corticotropes. POMC-B mRNA was not detectable in the corticotropes even following extended exposure times.

Key Findings, Chapter 4 - Gene Transcript Responses to Acute and Chronic Stress

- Results show for the first time that AVT transcripts in the parvocellular pre-optic region can be upregulated by a single two hour confinement stress, however, this upregulation is not a consistent response.
- Initial work on stress showed that AVT transcripts were elevated both four and eight hours after the start of an acute confinement stress.
- The magnocellular AVT neurones did not respond to any of the stressors tested.
- Although parvocellular CRH transcripts appear to rise in the absence of an AVT mRNA response following 5hrs confinement this increase was not significant.
- The upregulation of IT transcripts in the current study were difficult to interpret in themselves since plasma cortisol results indicate that an additional and undefined stress may have acted to raise transcripts during recovery.
- When either a mild (two minute low water) or severe (two hour confinement) stress was applied repeatedly for five or six days AVT gene transcripts declined to, or below, basal levels.
- The response of POMC mRNA in the pituitary melanotropes, which presumably reflects changes in release, varied between experiments. Thus, following confinement transcripts showed either no change, declined, or increased.
- Upregulation of POMC mRNA in the pituitary melanotropes, and an increase in plasma cortisol, occurred in the absence of an associated rise in POMC mRNA in the corticotropes in some instances.

Key Findings, Chapter 5 - Sensitivity of AVT and POMC mRNAs to Cortisol Negative Feedback

- Basal POMC transcripts in the corticotropes can be depressed when circulating concentrations of cortisol are raised for 48 hours above 60 ng/ml, the equivalent to that experienced during moderate chronic stress.
- AVT transcription was not significantly depressed until the highest cortisol treatment level of 2.3mg cortisol/kg body weight. This resulted in very high circulating cortisol levels, in excess

of those likely to be experienced during even severe stress. AVT gene transcripts returned to basal levels within two days of the last cortisol meal.

Key Findings, Chapter 6 - MCH mRNA Responses to Stress and the Possible HPI Modulatory Role of MCH

- Results suggest that the duration of stress does not necessarily determine the directional response of MCH transcripts. Thus, either a two minute low water, or two hour confinement stress results in a uniform lack of MCH mRNA response in either the NLT or LVR.
- When these stressors are applied repeatedly for five days, however, MCH transcripts in the NLT are downregulated; transcripts in the LVR remain unaffected. When administered daily for six days the two hour confinement fails to significantly reduce either NLT or LVR MCH mRNA.
- Results in this study show that the well documented modulatory effect of background elevated levels of MCH over the HPI axis only acts when the stress is mild.
- MCH transcripts in the NLT are unaffected by either acute low water (2 mins) or confinement (2 hrs). However, when low water is applied for one hour and combined with disturbance, MCH transcripts in the NLT increased. .
- In the current study both mild and more intense chronic stress reduced MCH mRNA in the NLT. Transcript responses in the LVR are, generally, in contrast to those seen in the NLT. Thus, following repeated two hour confinement or two minutes low water, MCH mRNA was downregulated in the NLT while remaining unchanged in the LVR.
- There was no clear difference in POMC levels in the corticotropes under basal or stress conditions.

The results show for the first time that AVT gene transcripts in the parvocellular neurones of the pre-optic nucleus can respond to acute stress. This lends weight to both the theory that these neurones are involved in the stress response, with the hypothalamic nuclei the PON in fish the homologue of the PVN stress center in mammals, and that these neurones are the source of the AVT fibres that abut on the corticotropes. However, the response of AVT to acute stress is not consistent, even when similar stressors are applied. The lack of a significant upregulation of transcripts in such instances may be attributable to a number of factors; it may be due to sampling fish at an inappropriate time after the stress, as it is possible that transcriptional responses are slower in trout than mammals. A recent preliminary study on the flounder found that AVT mRNA was significantly raised when measured twenty-four, but not four hours after confinement stress (Bond *et al.*, 2000). Furthermore, although AVT mRNA is downregulated in flounder following transfer, this does not occur until three days following the stress (Balment *et al.*, 2000).

Alternatively that AVT transcripts remain at basal following some stressors could be due to rapid negative feedback by cortisol. Our results suggest that although pharmacological

doses of cortisol are required to suppress AVT mRNA below basal levels, both AVT and POMC are able to respond to cortisol negative feedback. Unfortunately, since AVT transcripts were not increased in response to stress in these experiments, whether cortisol suppresses stress induced upregulation of AVT transcription could not be tested. Additional experiments are required in order to test the sensitivity of stress-induced rises of AVT and POMC mRNAs to cortisol negative feedback. In particular, increasing the duration of treatment would clarify whether AVT transcripts are responsive to extended periods of low exogenous plasma cortisol.

The response to stress of other ACTH-secretogogues, such as isotocin and CRH were also investigated. Unfortunately the lack of a reliable *in situ* hybridisation methodology for CRH did not allow a through investigation of its response to stress. Since it is a more potent ACTH secretogogue than AVT (Baker *et al.*, 1996) it is likely to play a more central role in this context. Results in the present study indicate that CRH mRNA may respond to acute stress and that this occurs in the absence of an AVT mRNA response. This is of particular interest and requires further investigation since the situation in fish may closely mimic that in rats in which there are selective controls over gene regulation for CRH and AVT (Ma *et al.*, 1997*a*, *b*). The addition of CRH data would thus serve to greatly augment the observations of the current study. The role of isotocin is likely to be less important than that of CRH; coupled with its low ACTH secretogogue effects in trout (Pierson *et al.*, 1996) the results suggest that isotocin may play a minor role in the response to stress. However, this may be a species specific phenomenon as isotocin is a more potent stimulator of ACTH release in the goldfish (Fryer *et al.*, 1985), and consequently may play a more important role in that species.

The possibility that there is more than one population of parvocellular AVT perikaryon in the PON with respect to where they terminate in the pituitary is difficult to ascertain. If not all the AVT neurones from this nucleus project to the corticotropes, it makes changes in their transcript abundance more difficult to interpret. That such distinct groups exist remains speculative, although comparisons of diurnal changes of parvo-AVT mRNA levels found in the current study versus those of circulating AVT found by other workers (Kulczykowska & Stolarski, 1996) suggest that this is a possibility since the two appear closely linked. Studies by other workers also indicate that AVT is released in response to a transfer stress (Balment *et al.*, 1993; Kulczykowska, 1997). Presumably AVT in this

instance could be being released by those parvocellular neurones that terminate in both the NIL and the corticotropes. Given that larger amounts of hormone are released into the blood, the contribution to the total transcript pool of those perikarya that release peptide near the corticotropes would be low. Thus, changes in transcription following stress would only be seen when AVT is released into the circulation by those parvocellular neurones that terminate in the NIL. Since plasma AVT peptide was not measured this could not be ascertained. Coupling transcript studies with plasma AVT measurements would clarify if significant increases in parvo-AVT mRNA are only seen when AVT peptide is released into the blood. In addition, use of retrograde transport studies would determine whether a proportion of the irAVT positive fibres in the NIL originate in the parvocellular region of the PON.

It is still unclear as to the degree of co-existence of CRH and AVT peptide in the parvocellular neurones. Immunostaining evidence in the current study suggests that there are many more CRH positive neurones than AVT. It is possible in the trout that, in contrast to the eel, all the parvo-neurones are CRH positive, and that a proportion of these also secrete AVT. Whether this is the case or not could be investigated using double fluorescence immunostaining together with confocal microscopy. If all the AVT neurones also secreted CRH, then one would presume that CRH is always be released during stress, with or without a synergist. Alternatively some neurones may contain only AVT. Consequently, during stress, AVT could be released in the absence of CRH. If CRH is the major POMC transcription-inducing peptide, as preliminary results suggest (personal communication; Dr.Tipping), then a failure to see a rise in POMC mRNA in the corticotropes after stress might be due to this. Stress may also be associated with changes in sensitivity of the corticotropes to CRH and AVT stimulation, such as occurs in rats (Hauger & Aguilera, 1992), and studies on pituitary responsiveness will ultimately be necessary in order to determine if this is the case.

In retrospect, if MCH influences the stress response, or a white background is in itself stressful, as results suggest, it might have been more instructive to have done parallel experiments on trout kept in grey tanks in which MCH release was not entirely suppressed though would not be exerting a strong modulatory influence. It would also have been useful to have more experimental data for black-reared fish, in parallel with those raised on white Transferring fish to a white background for shorter time periods,

such as a week, to observe the effect on the stress response of raised endogenous MCH, before any homeostatic adjustments to tank colour are made, would also have been instructive. If an immediate rise occurred in AVT and POMC mRNAs upon transfer to a white tank, this would also indicate that a white background is in itself stressful. If this is the case this could explain why some of the stressors applied failed to upregulate AVT transcripts, since AVT mRNA levels would presumably already be elevated as a consequence of the tank colour stress.

Varying the time of day when a stressor is applied can result in different magnitudes of the stress response, at least in rats. Thus, the restraint-induced rise in ACTH release varies dependant on whether the stressor is applied at the diurnal nadir or peak (Akana *et al.*, 1994). In the current study all experiments where carried out between 10.00h and 22.00h as this is when AVT transcripts plateau during the diurnal cycle. However, this plateau occurs at the peak of diurnal AVT mRNA levels. Since fish were kept under artificial lighting phase shifting the day would have been possible so that stressors could be applied during the aquarium night, when transcript levels are at their lowest. Whether this had an effect on the magnitude of the AVT mRNA response to either acute or chronic stress could be tested with such experiments. However, since chronic injection stress, applied daily either when MCH is high (10.30h) or lower (18.00h), has been shown to have no effect on the magnitude of cortisol released in trout (Green & Baker, 1991), whether the time of day when the stress is applied would result in differences in hypothalamic transcript responses is unclear.

Since all stressors used in the current work could be seen as psychological in nature it perhaps would have been interesting to observe AVT transcript responses following physiological stressors, particularly when applied repeatedly. Indeed, in the rat, differences in the response to chronically applied psychological as opposed to physiological stressors do occur (Herman *et al.*, 1996), which are thought to be mediated by the pathway by which the stress is perceived (Sawchenko *et al.*, 1993; Herman & Cullinan, 1997). Thus physiological stressors, such as injection of hypertonic saline, are perceived via pathways that run through the brain stem to the PVN, whilst psychological stressors, such as immobilisation, lead into the PVN via higher brain structures (Herman & Cullinan, 1997). If such different pathways exist in fish then it is possible that the

response to physiological stressors would have resulted in different responses of AVT mRNA to those psychological stressors predominantly used in the current study.

The importance of α-MSH in the control of cortisol release continues to be difficult to ascertain. The variability in the melanotrope POMC mRNA response to stress - either showing no change, increasing or declining following confinement - makes interpretation of results difficult. It is likely that α-MSH contributes to a mixture of corticosteroidogenic hormones released during stress, since *in vitro* evidence suggests that the control of cortisol release is multi-factorial and includes urotensin-1, angiotensin-II, and α-MSH (Arnold-Reed & Balment, 1994; Balm & Pottinger, 1995), as well as ACTH. The relative corticosteroidogenic contribution of α-MSH to this cocktail may be related to the intensity of the stress, though this requires further investigation.

Although additional information as to the response of specific transcripts, in particular AVT, to both acute and chronic stress has been gained a considerable body of work remains before the complex neuropeptide interactions of the HPI axis, particularly at the hypothalamic level, are thoroughly understood.

Appendix

Paraformaldehyde

One litre of phosphate buffered saline (PBS, pH 7.6) was heated to 50°C. To this 40g of paraformaldehyde (PFA) was added. With constant mixing concentrated NaOH (10M) was added drop-wise until the solution cleared. Following cooling to 4°C the pH was reduced to 7.6 using concentrated HCl (10M). The final solution (4% PFA, pH 7.6) was stored at 4°C for a maximum of three days prior to usage.

***In situ* Hybridisation Buffer**

The following solutions were prepared for use in the hybridisation buffer. All chemicals were obtained from Sigma.

20x SSC (Sodium Saline Citrate); 175.3g of sodium chloride and 88.2g of sodium citrate were added to 900mls of MilliQ water. This was mixed until all solid had dissolved. The resulting solution was made up to 1L and DEPC treated as described in Section 2.5.3. Lower concentrations of SSC (i.e. 1x and 2x SSC) were made up from this stock solution by diluting with DEPC treated MilliQ water.

Calf thymus DNA; 10mg/ml solution in DEPC treated water.

Yeast tRNA; 25mg/ml solution in DEPC treated water. It was necessary to sonicate the resulting mixture in order to dissolve the yeast tRNA. This was carried out on ice using ten second bursts until the yeast tRNA was fully dissolved. Aliquots of 0.5ml were stored at –20°C until use.

50x Denhardt's solution; 1g of ficoll, 1g of polyvinylpyrrolidone, and 1g of bovine serum albumen (BSA) were added to 100ml of DEPC treated MilliQ and mixed by inversion until dissolved. Aliquots of 0.5ml were stored at –20°C until required.

DTT (dithiothreotol); a 5M solution in DEPC treated MilliQ was made up then filtered through a sterile 0.2μm Acrodisk filter (Gelman Sciences, MI, USA). This was split into 50μl aliquots and stored at –20°C. Once thawed the solution could not be refrozen.

Dextran sulphate; a 50% solution was made up in DEPC treated MilliQ, thoroughly mixed, and stored at –20°C until required.

For the hybridisation mixture the following solutions were added to a 50ml Falcon tube in the given order at the volumes shown;

Formamide	25mls
20xSSC	10mls
Calf thymus DNA (10mg/ml)	2.5ml
Yeast tRNA (25mg/ml)	0.5ml
50x Denhardt's solution	1ml
Dextran sulphate (50%)	10mls
DEPC treated MilliQ	1ml

The final composition of the buffer was thus 50% formamide, 4xSSC (pH 7.2), 500μg/ml denatured calf thymus DNA, 250μg/ml yeast tRNA, 1x Denhardt's solution (0.02% polyvinylpyrolidone and 0.02% bovine serum albumen), 10% dextran sulphate (MW 500 000), and 500mM dithiothreitol.

Sublimated Bouin-Holland (BHS)

Saturated mercuric chloride; mercuric chloride was continually added to gently warmed distilled water until no further solid would dissolve. This was then cooled and stored at room temperature.

A solution of 4% picric acid, 10% formaldehyde, and 2.5% cupric acetate was made up in distilled water. To this mercuric chloride was added to 10% (v/v) immediately prior to use.

Table A.1; Details of parameter changes to the standard methdology for all CRH *in situ* hybridisations carried out. Probes were based on the sockeye salmon CRH gene sequence (Ando *et al.*, 1999). Two different probes were used. Probe 1 was complimentary to a region encoding for the mature peptide (from 5' – 3': cat gtc gaa cgt aag atc tag aga tat cgg). The other oligo, Probe 2, was complimentary to a region of the pre-CRH peptide sequence (from 5' – 3': gt cgt cga gct ggt tcg cga agc aga). Autoradiographic film was exposed for an initial period of six days. If no signal was visible at this stage a longer exposure of between two and four weeks was used. Activty/100µl is the final CPM (counts per minute) of the probe in 100µl of hybridisation mixture. Hybridisation and washing temperatures are quoted in °C. (f) in paranthesis denotes the use of frozen sections. Tick marks indicate a clearly visible positive signal on autoradiographic film; in all cases this was following six days of filom exposure. Cross marks indicate a lack of clearly discernable signal even after a four week exposure.

Trial No.	CRH Probe Sequence No	Activity/ 100µl	Hybridisation Temperature	Washing Temp.	Positive signal obtained?
Expt.2 (f)	1	$5x10^5$	37	60	✓
1	1	$5x10^5$	42	51.5	✓
2	1	$5x10^5$	42	55	✓
3	1	$5x10^5$	37	51.5	✓
4	1	$5x10^5$	37	55	✓
5	1	$3x10^4$	37	51.5	✗
2	1	$3x10^4$	42	51.5	✗
7	1	$6x10^4$	37	55	✗
8	1	$5x10^5$	37	50	✗
9	1	$5x10^5$	37	51.5	✗
10 (f)	1	$5x10^5$	37	51.5	✗
11 (f)	1	$5x10^5$	37	55	✗
12 (f)	1	$5x10^5$	37	60	✗
13	1	$5x10^5$	37	55	✗
14	1	$5x10^5$	42	51.5	✗
15	1	$5x10^5$	37	51.5	✗
16	2	$6x10^4$	37	50	✗
17	2	$5x10^5$	37	55	✗
18	2	$5x10^5$	37	51.5	✗
19	2	$5x10^5$	37	50	✗
20	2	$5x10^5$	42	51.5	✗
21	2	$5x10^5$	42	55	✗

Reference List

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Abe, K. J. K., Greer, M. A., and Critchlow, V. (1979). Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosteroid, body temperature, feeding and thyrotropin. *Neuroendocrinol.*, **29:** 119-131.

Abreu, M. E., Conti, L. H., Costello, D. G., and Enna, S. J. (1990). Corticotropin-releasing factor (CRF) and depression: behavioral, hormonal and receptor changes in rats following chronic administration of CRF. *Clin. Neuorpharmacol.*, **13:** 245-246.

Aguilera, G., Kiss, A., Hauger, R., and Tizabi, Y. (1992). 'Regulation of the hypothalamic-pituitary-adrenal axis during stress: role of neuropeptides and neurotransmitters. In: '*Stress: neuroendocrine and Molecular Approaches*'., pp. 365-381. Ed(s): R. Kvetnansky, R. McCarthy & J. Axelrod. New York: Gordon & Breach.

Akana, S. F., Strack, A. M., Hanson, E. S., and Dallman, M. F. (1994). Regulation of activity in the hypothalamus-pituitary-adrenal axis is integral to a larger hypothalamic system that determines caloric flow. *Endocrinol.*, **135:** 1125-1134.

Allison, C. M., and Omeljaniuk, R. J. (1998). Specific binding sites for [^{3}H]-dexamethasone in the hypothalamus of juvenile rainbow trout *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.*, **110:** 2-10.

Andersen, D. E., Reid, S. D., Moon, T. W., and Perry, S. F. (1991). Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.*, **48:** 1811-1817.

Ando, H., Hasegawa, M., Ando, J., and Urano, A. (1999). Expression of salmon corticotrophin-releasing hormone precursor gene in the preoptic nucleus in stressed rainbow trout. *Gen.Comp.Endocrinol.*, **113:** 87-95.

Antoni, F.A. (1986). Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocr. Rev.*, **7:** 351-357.

Antoni, F. A. (1993). Vasopressinergic control of pituitary adrenocorticotrophin secretion comes of age. *Front. Neuroendocrinol.*, **14:** 76-122.

Arends, R. J., Mancera, J. M., Munoz, J. L., and Wendelaar-Bonga, S. E. (1999). The stress response of gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *J.Endocrinol.*, **163:** 149-157.

Arends, R. J., Vermeer, H., Martens, G. J. M., Leunissen, J. A. M., Bonga, S. E. W., and Flik, G. (1998). Cloning and expression of two proopiomelanocortin mRNAs in the common carp (*Cyprinus carpio* L.). *Mol. Cell. Endocrinol.*, **143:** 23-31.

Arnold-Reed, D. E., and Balment, R. J. (1994). Peptide hormones influence *in vitro* secretion of cortisol in the trout *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.*, **96:** 85-91.

Baker, B. I. (1988). 'Relative importance of MCH and MSH in melanophore control.' In: *Advances in pigment cell research*, pp. 505-115. Ed(s): T. Bagnara. New York: Alan R Liss Inc.

Baker, B. I. (1991). Melanin-concentration hormone: a general vertebrate neuropeptide. *Int. Rev. Cytol.*, **126:** 1-47.

Baker, B. I. (1993). The role of melanin-concentrating hormone in colour change. *Ann. N.Y. Acad. Sci.*, **680:** 279-289.

Baker, B. I., and Bird, D. J. (1992). The biosynthesis of melanin-concentrating hormone in trout kept under different conditions of background colour and stress, as determined by an *in vitro* method. *J Neuroendocrinol.*, **4:** 673-679.

Baker, B. I., Bird, D. J., and Buckingham, J. C. (1985*a*). Salmonid melanin-concentrating hormone inhibits corticotrophin release. *J. Endocrinol.*, **106:** R5-R8.

Baker, B. I., Eberle, A. N., Baumann, J. B., Seigrist, W., and Girard, J. (1985*b*). Effect of melanin-concentrating hormone on pigment and adrenal cells *in vitro*. *Peptides,* **6:** 1125-1130.

Baker, B. I., Bird, D. J., and Buckingham, J. C. (1986). Effects of chronic administration of melanin-concentrating hormone on corticotrophin, melanotrophin, and pigmentation in the trout. *Gen .Comp. Endocrinol.*, **63:** 62-69.

Baker, B. I., Bird, D. J., and Buckingham, J. C. (1996). In the trout, CRH and AVT synergize to stimulate ACTH release. *Reg. Pep.*, **67:** 207-210.

Baker, B. I., Levy, A., Hall, L., and Lightman, S. L. (1995). Cloning and expression of melanin-concentrating hormone genes in the rainbow trout brain. *Neuroendocrinol.*, **61:** 67-76.

Baker, B. I., and Rance, T. A. (1981). Differences in concentrations of plasma cortisol in the trout and the eel following adaptation to black or white backgrounds. *J. Endocrinol.*, **89:** 135-140.

Balm, P. H. M., Gronveld, D., Lamers, A., and Wendelaar-Bonga, S. E. (1993). Multiple actions of melanotropic peptides in the teleost *Oreochromis mossambicus* (tilapia). *Ann. N.Y. Acad. Sci.*, **680:** 448-451.

Balm, P. H. M., and Pottinger, T. G. (1995). Corticotrophe and melanotrophe POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.*, **98:** 279-288.

Balment, R. J., Warne, J. M., Tierney, M., and Hazon, N. (1993). Arginine vasotocin and fish osmoregulation. *Fish Physiol. Biochem.*, **11:** 189-194.

Barber, L. D., Baker, B. I., Penny, J. C., and Eberle, A. N. (1987). Melanin-concentrating hormone inhibits the release of α–MSH from teleost pituitary glands. *Gen. Comp. Endocrinol.*, **65:** 79-86.

Barsyte, D., Tipping, D. R., Smart, D., Conlon, J. M., Baker, B. I., and Lovejoy, D. A. (1999). Rainbow trout (*Oncorhynchus mykiss*) urotensin-I: structural differences between urotensins-1 and urocortins. *Gen.Comp. Endocrinol.*, **115:** 169-177.

Bartanusz, V., Aubry, J.-M., Jezova, D., Baffi, J., and Kiss, J. Z. (1993*a*). Up-regulation of vasopressin mRNA in paraventricular hypophysiotrophic neurons after acute immobilisation stress. *Neuroendocrinol.*, **58:** 625-629.

Bartanusz, V., Jezova, D., Bertini, L. T., Tilders, F. J. H., Aubry, J. M., and Kiss, J. Z. (1993*b*). Stress-induced increase in vasopressin and corticotropin-releasing factor expression hypopysiotrophic paraventricular neurons. *Endocrinol.*, **132:** 895-902.

Bartanusz, V., Aubry, J.-M., Steimer, T., Baffi, J., and Kiss, J. Z. (1994). Stressor-specific increase in vasopressin mRNA in paraventricular hypophysiotrophic neurons. *Neurosci. Letters,* **170:** 35-38.

Barton, B. A., and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Ann. Rev. Fish Dis.*, **1:** 3-26.

Barton, B. A., Rahn, A. B., Feist, G., Bollig, H., and Schreck, C. B. (1998). Physiological stress responses of the freshwater chondrostean paddlefish (*Polyodon spathula*) to acute physical disturbances. *Comp. Biochem. Physiol. A,* **120:** 355-363.

Barton, B. A., Schreck, C. B., and Barton, L. D. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions and stress responses in juvenile rainbow trout. *Dis. Aquat. Organisms,* **2:** 173-185.

Berkenbosch, F., Tilders, F. J. H., and Vermes, I. (1983). Beta-adrenoreceptor activation mediates stress-induced secretion of beta-endorphin related peptides from the intermediate but not the anterior pituitary. *Nature,* **305:** 237-239.

Berkenbosch, F., Vermes, I., and Tilders, F. J. H. (1984). The β-adrenoreceptor blocking drug propranolol prevents secretion of immunoreactive β-endorphin and α-MSH to certain stress stimuli. *Endocrinol.,* **115:** 1051-1059.

Bernier, N. J., Li, X., and Peter, R. E. (1999). Differential expression of corticotropin-releasing factor (CRF) and urotensin I precursor genes, and evidence of CRF gene expression regulated by cortisol in goldfish brain. *Gen. Comp. Endocrinol.,* **116:** 461-477.

Berridge, C. W., and Dunn, A. J. (1986). Corticotropin-releasing factor elicits naloxone sensitive stress-like alterations in exploratory behaviour in mice. *Regul.Pept.,* **16:** 83-93.

Birnberg, N. C., Lissitzky, J.-C., Hinman, M., and Herbert, E. (1983). Glucocorticoids regulate proopiomelanocortin gene expression *in vivo* at the levels of transcription and secretion. *Proc. Natl. Acad. Sci. USA,* **80:** 6982-6986.

Bittencourt, J. C., and Sawchenko, P. E. (2000). Do centrally administered neuropeptides access cognate receptors? An analysis in the central corticotropin-releasing factor system. *J.Neurosci.,* **20:** 1142-1156.

Bond, H., Warne, J., and Balment, R. 'Hypothalamic-Pituitary-Interrenal-Axis (HPIA) activation leads to a change in pro-VT mRNA expression in the pre-optic area (POA) of the flounder.' 4th Int. Sym. Fish Endocrinol., 2000; P144.

Bradbury, M. J., Cascio, C. S., Scribner, K. A., and Dallmann, M. F. (1991). Stress-induced adrenocorticotropin secretion: Diurnal responses and increases during stress in the evening are not dependent on corticosterone. *Endocrinol.,* **128:** 680-688.

Buckingham, J. C. (1996). Stress and the neuroendocrine-immune axis: the pivotal role of glucocorticoids and liopcortin 1. *British J. Pharmacol.*, **118:** 1-19.

Buckingham, J. C., Smith, T., and Loxley, H. D. (1992). 'The control of ACTH secretion.' In: *Comprehensive Endocrinology*, pp. 131-158. Ed(s): V. H. T. James. New York: Raven Press.

Cai, A., Scarbrough, K., Hinkle, D., and Wise, P. (1997). Fetal grafts containing chiasmatic nuclei restore diurnal rhythm of CRH and POMC mRNA in aging rats. *Am. J. Physiol.*, **273:** 1764-1770.

Cambre, M. L., Verdonck, W., Ollevier, F., Vandesande, F., Batten, T. F. C., and Kuhn, E. R. (1986). Immunocytochemical identification and localisation of the different cell types in the pituitary of the sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.*, **61:** 368-375.

Chakraborti, P. K., Weisbart, M., and Chakraborti, A. (1987). The presence of corticosteroid receptor activity in the gills of the Brook trout, *Salvelinus fontinalis. Endocrinol.*, **66:** 323-332.

Chan, D. K. O., and Woo, N. Y. S. (1978). Effect of cortisol on the metabolism of the eel, *Anguilla japonica. Gen. Comp. Endocrinol.*, **35:** 205-215.

Chauveau, I., Salbert, G., Bunel, D. T., Vaudry, H., Valotaire, Y., and Jego, P. (1993). Cloning and sequencing of two POMC cDNAs in rainbow trout (*Oncorhynchus mykiss*): effect of sex steroids on the expression of the POMC genes. *Proc. Natl. Acad. Sci. USA,* **680:** 476-477.

Chrisitan, H. C., Goulding, N. J., Kahan, M., Wang, H., Morris, J. F., Flower, R. J., and Buckingham, J. C. (1996). Detection of lipocortin 1 (LC1) binding sites in the rat anterior pituitary gland by fluorescent activated cell analysis/Sorting (FCAS). *Br. J. Pharmacol.*, **119:** 61.

Christian, H. C., Taylor, A. D., Flower, R. J., Morris, J. F., and Buckingham, J. C. (1997). Characterisation and localisation of lipocortin-1 binding sites on rat anterior pituitary cells by fluorescence-activated cell analysis/sorting and electron microscopy. *Endocrinol.*, **138:** 5341-5351.

Cummins, S., Elde, R., and Lindall, A. (1983). Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat. *J. Neurosci.*, **3:** 1355-1368.

Dallman, M. F. (1993). Stress Update - adaptation of the hypothalamic-pituitary-adrenal axis to chronic stress. *T.E.M.*, **4:** 62-69.

Dallman, M. F., Demanicore, D., and Shinsako, J. (1974). Diminishing corticotrope capacity to release ACTH during sustained stimulation: the twenty-four hours after bilateral adrenalectomy. *Endocrinol.*, **95:** 65.

Darlington, D. N., Barraclough, C. A., and Gann, D. S. (1992). Hypotensive hemorrhage elevates corticotropin-releasing hormone messenger ribonucleic acid (mRNA) but not vasopressin mRNA in the rat hypothalamus. *Endocrinol.*, **130:** 1281-1288.

Davis, K. B., Torrance, P., Parker, N. C., and Suttle, M. A. (1985). Growth, body composition and hepatic tyrosine amino-transferase activity in cortisol fed channel catfish, *Ictalurus punctatus* Rafinesque. *J. Fish Biol.*, **27:** 177-184.

Davis, L. G., Arentzen, R., Reid, J. M., Manning, R. W., Wolfson, B., Lawrence, K. L., and Baldino, F. (1986). Glucocorticoid sensitivity of vasopressin mRNA levels in the paraventricular nucleus of the rat. *Proc. Natl. Acad. Sci., USA*, **83:** 1145-1149.

Davis, R. E. (1983). 'Higher brain areas and functions.' In: *Fish Neurobiology*, Michigan: University of Michigan Press.

De Groeij, D. E. C., Jezova, D., and Tilders, F. J. H. (1992). Repeated stress enhances vasopressin synthesis in corticotropin-releasing factor neurons in the paraventricular nucleus. *Brain Res.*, **577:** 165-168.

De Groeij, D. E. C., Kvetnansky, R., Whitnall, M. H., Jezova, D., Berkenbosch, F., and Tilders, F. J. H. (1991). Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and co-localisation with corticotropin-releasing factor in the median eminence of rats. *Neuroendocrinol.*, **53:** 150-159.

De Pedro, N., Alonso-Gomez, A. L., Gancedo, B., Delgado, M. J., and Alonso-Bedate, M. (1993). Role of corticotropin-releasing factor (CRF) as a food intake regulator in goldfish. *Physio. Behav.*, **53:** 517-520.

De Pedro, N., Gancedo, B., Alonso-Gomez, A. L., Delgado, M. J., and Alonso-Bedate, M. (1995). Alterations in food intake and thyroid tissue content by corticotropin-releasing factor in *Tinca tinca*. *Rev. Esp. Fisiol.*, **51:** 71-75.

De Pedro, N., Pinillos, M. L., Valenciano, A. I., Alons-Bedate, M., and Delgado, M. J. (1998). Inhibitory effects of serotonin on feeding behaviour in goldfish: Involvement of CRF. *Peptides,* **19:** 505-511.

DeSouza, E. B., Insel, T. R., Perrin, M. H., J., R., and Vale, W. W. (1985). Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: an autoradiographic study. *J. Neurosci.*, **5:** 3189-3203.

Ducouret, B., Tujague, M., Ashraf, J., Mouchel, N., Servel, N., Valotaire, Y., and Thompson, E. B. (1995). Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. *Endocrinol.*, **136:** 3774-3783.

Dunn, A. J., and File, S. E. (1987). Corticotropin-releasing factor has an anxiogenic action in the social interaction test. *Horm. Behav.*, **21:** 193-202.

Eberwine, J. H., and Roberts, J. L. (1984). Glucocorticoid regulation of proopiomelanocortin gene transcription in the rat pituitary. *J. Biol. Chem.*, **259:** 2166-2170.

Ekstrom, P., Meissl, H. (1997). The pineal organ of teleost fishes. *Rev. Fish Biol. Fish.*, **7:** 199-284.

Eipper, B. A., and Mains, R. E. (1980). Structure and function of proadrenocorticotropin/endorphin and related peptides. *Ann. Rev. Biochem.*, **53:** 665-715.

Ellsaesser, C. F., and Clem, L. W. (1986). Hematological and immunological changes in channel catfish stressed by handling and transport. *J. Fish Biol.*, **28:** 511-521.

Esch, G. W., and Hazen, T. C. (1980). Stress and body condition in a population of largemouth bass; implications for red-sore disease. *Trans. Am. Fish. Soc.*, **109:** 532-536.

Farbridge, K. J., and Leatherland, J. F. (1986). A comparative immunohistochemical study of the pars distalis in six species of teleost fishes. *Fish Physiol. Biochem.*, **1:** 63-74.

Flower, R. J. (1988). Lipocortin 1 and the mechanisms of action of the glucocorticoids. *Br. J. Pharmacol.*, **94:** 987-1015.

Foran, C. M., and A.H., B. (1999). Preoptic GnRH and AVT: Axes for sexual plasticity in teleost fish. *Gen. Comp. Endocrinol.*, **116:** 141-152.

Foran, C. M., and Bass, A. H. (1998). Preoptic AVT immunoreactive neurons of a teleost fish with alternative reproductive tactics. *Gen. Comp. Endocrinol.*, **111:** 271-282.

Francis, K. F. (1996). 'The distribution and functions of MCH in lower vertebrates.' *Department of Biology and Biochemistry*, University of Bath, Bath.

Francis, K., Suzuki, M., and Baker, B. I. (1997). Responses of melanin-concentrating hormone mRNA to salt water challenge in the rainbow trout. *Neuroendocrinol.*, **66:** 195-202.

Freeman, H. C., and Idler, D. R. (1973). Effects of corticoids on liver trans-aminases in two salmonids, the rainbow trout (*Salmo gairdneri*) and the brook trout (*Salvelinus fontinalis*). *Gen. Comp. Endocrinol.*, **20:** 69-75.

Fryer, J., Lederis, K., and Rivier, J. (1984). Cortisol inhibits the ACTH-releasing activity of urotensin 1, CRF, and sauvagine as observed with superfused goldfish pituitary cells. *Peptides,* **5:** 925-930.

Fryer, J., Lederis, K., and Rivier, J. (1985). ACTH-releasing activity of urotensin I and ovine CRF: interactions with arginine vasotocin, isotocin and arginine vasopressin. *Reg Peptides,* **11:** 11-15.

Fryer, J. N., and Lederis, K. (1988). 'Comparison of actions of posterior pituitary hormones on corticotropin secretion in mammals and fishes.' Satellite Symposium. VII International Congress of Endocrinology, Posterior Pituitary Hormones., Ed(s): Kyoto.

Fryer, J. N., and Peter, R. E. (1975). Stress and adrenocorticosteroid dynamics in the goldfish. *Canad. J. Zool.*, **53:** 1012-1020.

Fryer, J. N. (1989). Neuropeptides regulating the activity of goldfish corticotropes and melanotropes. *Fish Physiol.Biochem.*, **7:** 21-27.

Garcia-Belenguer, S., Oliver, C., and Mormede, P. (1993). Facilitation and feedback in the hypothalamo-pituitary-adrenal axis during food restriction in rats. *J. Neuroendocrinol.*, **5:** 663-668.

Gilchriest, B. J., Tipping, D. R., Levy, A., and Baker, B. I. (1998). Diurnal changes in the expression of genes encoding for arginine vasotocin and pituitary pro-opiomelanocortin in the rainbow trout (*Oncorhynchus mykiss*): correlation with changes in plasma hormones. *J. Neuroendocrinol.*, **10:** 937-943.

Gilham, I. D., and Baker, B. I. (1985). A black background facilitates the response to stress in teleosts. *J. Endocrinol.*, **105:** 99-105.

Gillies, G. E., Linton, E. A., and Lowry, P. J. (1982). Corticotrophin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature,* **299:** 355-357.

Gomez, F., Lahmame, A., De Kloet, E. R., and Armario, A. (1996). Hypothalamic-pituitary-adrenal response to chronic stress in five inbred rat strains: differential response are mainly located at the adrenocorticol level. *Neuroendocrinol.,* **63:** 327-337.

Green, J. A., and Baker, B. I. (1991). The influence of repeated stress on the release of melanin-concentrating hormone in the rainbow trout. *J. Endocrinol.,* **128:** 261-266.

Green, J. A., Baker, B. I., and Kawauchi, H. (1991). The effect of rearing rainbow trout on black and white backgrounds on their secretion of melanin-concentrating hormone and their sensitivity to stress. *J. Endocrinol.,* **128:** 267-274.

Groneveld, D., Balm, P. H. M., Martens, G. J. M., and Wendelaar Bonga, S. E. (1995). Differential melanin-concentrating hormone gene expression in two hypothalamic nuclei of the teleost tilapia in response to environmental changes. *J.Neuroendocrinol.,* **7:** 527-533.

Harbuz, M. S., Jessop, D. S., Lightman, S. L., and Chowdrey, H. S. (1994). The effect of restraint or hypertonic saline stress on corticotropin-releasing factor, arginine vasopressin, and proenkephalin A mRNAs in the CFY, Sprague and Wistar strains of rat. *Brain Res.,* **667:** 6-12.

Harbuz, M. S., and Lightman, S. L. (1989*a*). Glucocorticoid inhibition of stress-induced changes in hypothalamic corticotropin-releasing factor messenger RNA and proenkephalin A messenger RNA. *Neuropep.,* **14:** 17-20.

Harbuz, M. S., and Lightman, S. L. (1989*b*). Responses of hypothalamic and pituitary mRNA to physical and psychological stress in the rat. *J. Endocrinol.,* **122:** 705-711.

Harbuz, M. S., and Lightman, S. L. (1992). Stress and the hypothalamo-pituitary-adrenal axis: acute, chronic and immunological activation. *J. Endocrinol.,* **134:** 327-339.

Harbuz, M. S., Rees, R. G., Eckland, D., Jessop, D. S., Brewerton, D., and Lightman, S. L. (1992). Paradoxical responses of hypothalamic CRF mRNA and CRF-41 peptide and adenohypophysial POMC mRNA during chronic inflammatory stress. *Endocrinol.*, **130:** 1394-1400.

Hashimoto, K., Suemaru, S., Takao, T., Sugarwara, M., Makino, S., and Ota, S. (1988). Corticotropin-releasing hormone and pituitary-adrenocortical responses in chronically stressed rats. *Reg. Pept.*, **23:** 117-126.

Hatalski, C. G., Guirguis, C., and Baram, T. Z. (1998). Corticotropin-releasing factor mRNA expression in the hypothalamic paraventricular nucleus and the central nucleus of the amygdala is modulated by repeated acute stress in the immature rat. *J. Neuroenodcrinol.*, **10:** 663-670.

Hauger, R. I., Millan, M. A., Lorang, M., Harwood, J. P., and Aguilera, G. (1988). Corticotropin-releasing factor receptor and pituitary adrenal responses during mobilisation stress. *Endocrinol.*, **123:** 396-405.

Hauger, R. I., Millan, M. A., Lorang, M., Irwin, M., and Aguilera, G. (1990). CRF receptor regulation and sensitisation of ACTH responses to acute stress during chronic stress intermitted immobilisation stress. *Brain Res.*, **532:** 34-40.

Hauger, R. L., and Aguilera, G. (1992). Regulation of corticotropin-releasing factor hormone receptors and hypothalamic pituitary-adrenal axis responsiveness during cold stress. *J. Neuroendocrinol.*, **4:** 617-624.

Herman, J. P., Schafer, M. K.-H., Young, E. A., Thompson, R., Douglas, J., Akil, H., and Watson, S. J. (1989). Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis. *J. Neurosci.*, **9:** 3072-3078.

Herman, J. P., Adams, D., and Prewitt, C. (1995). Regulatory changes in neuroendocrine stress-integrative circuitry produced by variable stress paradigm. *Neuroendocrinol.*, **61:** 180-190.

Herman, J., Prewitt, C., and Cullinan, W. (1996). Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis. *Crit. Revs. Neurobiol.*, **10:** 371-394.

Herman, J. P. (1995). *In situ* hybridisation analysis of vasopressin gene transcription in the paraventricular and supraoptic nuclei of the rat: regulation by stress and glucocorticoids. *J. Comp. Neurol.*, **363:** 15-27.

Herman, J. P., and Cullinan, W. E. (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenal axis. *Trends in Neurosci.*, **20:** 78-84.

Hiraoka, S., Suzuki, M., Yanagisawa, T., Iwata, M., and Urano, A. (1993). Divergence of gene expression in neurohypophysial hormone precursors among salmonids. *Gen Comp. Endocrinol.*, **92:** 1-10.

Hiroshige, T., and Sakakura, M. (1971). Circadian rhythm of corticotrophin-releasing activity in the hypothalamus of normal and adrenalectomised rats. *Neuroendocrinol.*, **7:** 25-36.

Hlaveck, R. R., and Bulkley, R. V. (1980). Effects of malachite green on leukocyte abundance in rainbow trout, *Salmo gairdneri* (Richardson). *J. Fish Biol.*, **17:** 431-444.

Hobby, A. C., Pankhurst, N. W., and Haddy, J. A. (2000). The effect of short term confinement stress on binding characteristics of sex steroid binding protein (SBP) in female black bream (*Acanthopagrus butcheri*) and rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. A*, **125:** 85-94.

Hontela, A., and Lederis, K. (1985). Diel variations in arginine vasotocin content of goldfish brain and pituitary: effects of photoperiod and pinealectomy. *Gen.Comp.Endocrinol.*, **57:** 397-404.

Hontela, A., and Peter, R. (1980). Effects of pinealectomy, blinding, and sexual condition on serum gonadotrophin levels in the goldfish. *Gen. Comp. Endocrinol.*, **40:** 168-179.

Hontela, A., Rasmussen, J. B., Ko, D., Lederis, K., and Chevalier, G. (1991). Arginine vasotocin, an osmoregulatory hormone as a potential indicator of acid stress in fish. *Can.J.Fish.Aquat.Sci.*, **48:** 238-242.

Hontela, A., Rasmussen, J. B., Lederis, K., Tra, H. V., and Chevalier, G. (1993). Elevated levels of arginine vasotocin in the brain of brook trout (*Salvelinus fontinalis*) from acid lakes: a field test of a potential biomarker for acid stress. *Can.J.Aquat.Sci.*, **50:** 1717-1727.

Hyodo, S., Kato, Y., Ono, M., and Urano, A. (1991). Cloning and sequence analyses of cDNAs encoding vasotocin and isotocin precursors of chum salmon, *Oncorhynchus keta*: evolutionary relationships of neurohypophysial hormone precursors. *J.Comp.Physiol. B,* **160:** 601-608.

Hyodo, S., and Urano, A. (1991). Changes in expression of provasotocin and proisotocin genes during adaptation to hyper- and hypo-osmotic environments in rainbow trout. *J. Comp. Physiol. B,* **161:** 549-556.

Idler, D. R., and Truscott, B. (1972). 'Corticosteroids in fish.' In: *Steroids in non-mammalian vertebrates*, pp. 127-252. Ed(s): D. R. Idler. London: Academic Press.

Imaki, T., Shibasaka, T., Hotta, M., and Demura, H. (1992). Early induction of c-fos ribonucleic acid in the paraventricular nucleus after immobilization stress. *Endocrinol.*, **132:** 240-246.

Ishii, S., and Urano, O. (1980). 'Neurosecretion.' Tokyo, University of Tokyo Press.

Kalin, N. H., Takahashi, L. K., and Chen, F.-L. (1994). Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus. *Brain Res.*, **656:** 182-186.

Kant, G. J., Eggleston, T., Landman-Roberts, L., ?, K., Driver, G. C., and Meyerhoff, J. L. (1985). Habituation to repeated stress is stressor specific. *Pharmacol., Biochem., & Behav.*, **22:** 631-634.

Kaul, S., and Vollrath, L. (1974). The goldfish pituitary. I: Cytology. *Cell Tiss. Res.*, **154:** 231-249.

Kavaliers, M. (1979). The pineal organ and circadian organization of teleost fish. *Rev. Canad. Biol.*, **38:** 281-292.

Kawauchi, H. (1983). Chemistry of proopiocortin-related peptides in the salmon pituitary. *Arch. Biochem. Biophys.*, **227:** 343-350.

Keller-Wood, M. E., and Dallman, M. F. (1984). Corticosteroid inhibition of ACTH secretion. *Endocrin. Rev.*, **5:** 1-25.

Kelsall, C. J., and Balment, R. J. (1995). Effects of native urotensins on *in vitro* cortisol secretion in the flounder. *J. Endocrinol.*, **143:** 15.

Kelsall, C. J., and Balment, R. J. (1998). Native urotensins influence cortisol secretion and plasma cortisol concentrations in the euryhaline flounder, *Platichthys flesus*. *Gen. Comp. Endocrinol.*, **112:** 210-219.

Kishida, M., Baker, B. I., and Bird, D. J. (1988). Localisation and identification of melanocyte-stimulating hormones in the fish brain. *Gen. Comp. Endocrinol.*, **71:** 229-242.

Kishida, M., Baker, B. I., and Eberle, A. N. (1989). The measurement of melanin-concentrating hormone in trout blood. *Gen. Comp. Endocrinol.*, **74:** 221-229.

Kiss, J. Z., Mezey, E., and Skirboll, L. (1984). Corticotropin-releasing factor-immunoreactive neurons of the paraventricular nucleus become vasopressin positive after adrenalectomy. *Proc. Natl. Acad. Sci. USA*, **81:** 1854-1858.

Kitraki, E., Karandrea, D., and Kittas, C. (1999). Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain. *Neuroendocrinol.*, **69:** 331-338.

Klinger, H., Delventhal, H., and Hilge, V. (1983). Water quality and stocking density as stressors of channel catfish (*Ictalurus punctatus* Raf.). *Aquaculture*, **30:** 263-272.

Knoebl, I., Fitzpatrick, M. S., and Schreck, C. B. (1996). Characterisation of a glucocorticoid receptor in the brains of chinook salmon, *Oncorhynchus tshawytscha*. *Gen. Comp. Endocrinol.*, **101:** 195-204.

Koob, G. F., and Heinrichs, S. C. (1999). A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res.*, **848:** 141-152.

Krieger, D. T. (1975). Circadian pituitary adrenal rhythms. *Adv. Exp. Med. Biol.*, **54:** 169-189.

Krieger, A. T., and Hauser, H. (1978). Comparison of synchronization of circadian corticosteroid rhythms by photoperiod and food. *Proc.Soc.Natl.Acad.Sci., USA,* **75:** 1577-1581.

Krieger, D. T., and Luria, M. (1977). Plasma ACTH and cortisol responses to TRF, vasopressin or hypoglycemia in Cushing's disease and Nelson's syndrome. *J Clin Endocrinol Metab,* **44:** 361-368.

Kulczykowska, E. (1997). Response of circulating arginine vasotocin and isotocin to rapid osmotic challenge in rainbow trout. *Comp. Biochem. Physiol. A,* **118:** 773-778.

Kulczykowska, E., and Stolarski, J. (1996). Diurnal changes in plasma arginine vasotocin and isotocin in rainbow trout adapted to fresh water and brackish Baltic water. *Gen. Comp. Endocrinol.*, **104:** 197-202.

Kwak, S. P., Young, A. E., Akil, H., and Watson, S. J. (1992). Changes in pro-opiomelanocortin primary transcript levels in the anterior pituitary accompany increased adrenocorticotropin secretion during diurnal surge. *J. Neuroendocrinol.*, **4:** 21-27.

Kwak, S. P., Morano, M. I., Young, E. A., Watson, S. J., and Akil, H. (1993). Diurnal CRHmRNA rhythm in the hypothalamus: Decreased expression in the evening is not dependent on endogenous glucocorticoids. *Neuroendocrinol.*, **57:** 96-105.

Lamers, A. E., Flik, G., Atsma, W., and Bonga, S. E. W. (1992). A role for di-acetyl alpha-melanocyte stimulating hormone in the control of cortisol release in the teleost *Oreochromis mossambicus*. *J. Endocrinol.*, **135:** 285-292.

Larsen, D. A., Swanson, P., Dickey, J. T., Rivier, J., and Dickhoff, W. W. (1998). *In vitro* thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.*, **109:** 276-285.

Le Mevel, J.-C., Pamantung, T.-F., Mabin, D., and Vaudry, H. (1993). Effects of central and peripheral administration of arginine vasotocin and related neuropeptides on blood pressure and heart rate in the conscious trout. *Brain Res.*, **610:** 82-89.

Lederis, K., Fryer, J. N., Okawara, Y., Schonrock, C., and Richter, D. (1994). 'Corticotropin-releasing factors acting on the fish pituitary: experimental and molecular analysis.' In: *Fish Physiology*, pp. Ed(s): N. M. Sherwood & C. Hew. San Diego: Academic Press.

Lee, P. C., Goodrich, M., Struve, M., Yoon, H. L., and Weber, D. (1992). Liver and brain glucocorticoid receptor in rainbow trout *Oncorhynchus mykiss*: downregulation by dexamethasone. *Gen. Comp. Endocrinol.*, **87:** 222-231.

Levin, N., Blum, R., and Roberts, J. L. (1989). Modulation of basal and corticotropin-releasing factor-stimulated proopiomelanocortin (POMC) gene expression by vasopressin in rat anterior pituitary. *Endocrinol.*, **125:** 2957-2966.

Levin, N., and Roberts, J. L. (1991). Positive regulation of proopiomelanocortin gene expression in corticotropes and melanotropes. *Front. Neuroendocrinol.*, **12:** 1-22.

Lightman, S. L., and Harbuz, M. S. (1993). 'Expression of corticotropin releasing factor mRNA in response to stress.' In: *Corticotropin-Releasing Factor*, pp. 173-188. Ed(s): Chichester: Wiley.

Lightman, S. L., and Young III, W. S. (1988). Corticotropin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. *J.Physiol.*, **403:** 511-523.

Lightman, S. L., and Young III, W. S. (1989). Influence of steroids on the hypothalamic corticotrophin-releasing factor and preproenkephalin mRNA responses to stress. *Proc. Natl. Acad. Sci., USA,* **86:** 4306-4310.

Linton, E. A., Tilders, F. J. H., Hodgkinson, S., Birkenbosch, F., Vermes, I., and Lowry, P. J. (1985). Stress-induced secretion of adrenocorticotropin in rats is inhibited by administration of antisera to ovine corticotropin-releasing factor and vasopressin. *Endocrinol.*, **116:** 966-970.

Liu, J. P., Robinson, P. J., Funder, J. W., and Engler, D. (1990). The biosynthesis and secretion of adrenocorticotropin by the ovine anterior pituitary is predominantly regulated by arginine vasopressin (AVP) - evidence that protein kinase-C mediates the action of AVP. *J. Biol. Chem.*, **265:** 14136-14142.

Lovejoy, D. A., and Balment, R. J. (1999). Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. *Gen. Comp. Endocrinol.*, **115:** 1-22.

Lyon, V. E., and Baker, B. I. (1993). The effect of photoperiod on plasma levels of melanin-concentrating hormone in the trout. *J Neuroendocrinol.*, **5:** 193-499.

Ma, X.-M., Levy, A., and Lightman, S. L. (1997*a*). Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotrophin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinol.*, **138:** 4351-4357.

Ma, X.-M., Levy, A., and Lightman, S. L. (1997*b*). Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *J.Endocrinol.*, **152:** 81-89.

Ma, X.-M., Levy, A., and Lightman, S. L. (1997*c*). Rapid changes of heteronuclear RNA for arginine vasopressin but not for corticotropin releasing hormone in response to acute corticosterone administration. *J. Neuroendocrinol.*, **9:** 723-728.

Madsen, G., Chan, E. C., Falconer, J., Ho, K. Y., and Smith, R. (1991). Reverse hemolytic plaque assay study of corticotropin-releasing hormone and arginine vasopressin interaction in the ovine corticotropes. *J.Neuroendocrinol.*, **3:** 193-197.

Makino, S., Smith, M. A., and Gold, P. W. (1995). Increased expression of corticotropin-releasing factor hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinol.*, **136:** 3299-3309.

Malo-Michele, M. (1980). Stimulation of the cortico-melanotropes of the pars intermedia of the hypophysis of Saupe, *Boops salpa* L. (marine teleost) subjected to a noise stress. *Gen. Comp. Endocrinol.*, **42:** 101-104.

Mamalaki, E., Kvetnansky, R., Brady, L. S., Gold, P. V., and Herkenham, M. (1992). Repeated immobilisation stress alters tyrosine hydroxylase, corticotropin-releasing hormone and corticosteroid receptor messenger ribonucleic acid levels in rat brain. *J. Neuroendocrinol.*, **4/5:** 689-699.

Mancera, J. M., and Fernadez-Llebrez, P. (1995). Localization of corticotrophin-releasing factor immunoreactivity in the brain of the teleost *Sparus aurata. Cell Tissue Res.*, **281:** 569-572.

Matz, S. P., and Hofeldt, T. (1999). Immunohistochemical localization of corticotropin-releasing factor in the brain and thyrotropin stimulating hormone in the pituitary of chinook salmon (*Oncorhynchus tshawytscha*). *Gen. Comp. Endocrinol.*, **114:** 151-160.

McBride, J. R., and van Overbeeke, A. P. (1971). Effects of androgens, estrogens, and cortisol on the skin, stomach, liver, pancreas, and kidney in gonadectomised adult sockeye salmon (*Oncorhynchus nerka*). *J. Fish. Res. Bd Can.*, **28:** 485-490.

Metcalfe, N. B., Huntingford, F. A., Graham, W. D., and Thorpe, J. E. (1989). Early social status and the development of early life-history strategies in Atlantic salmon. *Proc. Royal Soc. Lon. B,* **236:** 7-19.

Micale, V., Maricchiolo, G., and Genovese, L. (1999). The reproductive biology of the amberjack, *Seriola dumerilii* (Risso 1810). I. Oocyte development in captivity. *Aquacult. Res.,* **30:** 349-355.

Minth, C., Qiu, H., Akil, H., Watson, S. J., and Dixon, J. E. (1989). Two precursors of melanin-concentrating hormone: DNA sequence analysis and *in situ* and immunochemical localization. *Proc. Natl. Acad. Sci., USA,* **86:** 4292-4296.

Mommsen, T. P., Vijayan, M. M., and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. and Fish.,* **9:** 211--268.

Momose, K., Inui, A., Asakawa, A., Ueno, N., Nakajima, M., Fujimiya, M., and Kasuga, M. (1999). Intracerebroventricularly administered corticotropin-releasing factor inhibits food intake and produces anxiety-like behaviour at very low doses in mice. *Diabetes, Obesity & Metab.,* **1:** 281-284.

Morehead, D. T. (1998). Effect of capture, confinement and repeated sampling on plasma steroid concentrations and oocyte size in female striped trumpeter *Latris lineata* (Latrididae). *Marine Fresh. Res.,* **49:** 373-377.

Murphy, D., Costall, B., and Smythe, J. W. (1998). Regulation of hippocampal theta activity by corticosterone: opposing functions of mineralocorticoid and glucocorticoid receptors. *Brain Res. Bull.,* **45:** 631-635.

Nahon, J.-L., Presse, F., Schoepfer, R., and Vale, W. (1991). Identification of a single melanin-concentrating hormone messenger ribonucleic acid in coho salmon: structural relatedness with 7SL ribonucleic acid. *J Neuroendo,* **3:** 173-183.

Nakai, Y., Usui, T., Tsukada, T., Takahashi, H., Fukata, J., Fukushima, M., Senoo, K., and Imura, H. (1991). Molecular mechanisms of glucocorticoid inhibition of human proopiomelanocortin gene-transcription. *J. Steroid Biochem. Mol. Biol.*, **40:** 301-306.

Nandir, J., and Bern, H. A. (1960). Corticosteroid production by interrenal tissue of teleost fishes. *Endocrinol.*, **66:** 295-303.

Nandir, J., and Bern, H. A. (1965). Chromatography of corticosteroids from teleost fishes.. *Gen. Comp. Endocrinol.*, **5:** 1-15.

Negri, L., Noviello, L., and Noviello, V. (1985). Effects of sauvagine, urotensin 1 and CRF on food intake in rats. *Peptides*, **6 (Suppl):** 53-57.

Nojiri, H., Sato, M., and Urano, A. (1986). Increase in the vasopressin mRNA levels in the magnocellular neurosecretory neurons of water-deprived rats: *in situ* hybridization study with the use of synthetic oligonucleotide probe. *Zool. Sci.*, **3:** 345-350.

Nolan, D. T., Veld, R. L. J. M. O., Balm, P. H. M., and Wendelaar Bonga, S. E. W. (1999). Ambient salinity modulates the response of the tilapia, *Oreochromis mossambicus* (Peters), to net confinement. *Aquacult.*, **177:** 297-309.

Okawara, Y., Ko, D., Morley, S. D., Richter, D., and Lederis, K. P. (1988). Cloning and sequence analysis of cDNA for corticotropin-releasing factor precursor from the teleost fish *Catostomus commersoni*. *Proc. Natl. Acad. Sci. USA*, **85:** 8439-8443.

Okawara, Y., Ko, D., Morley, S. D., Richter, D., and Lederis, K. P. (1992). *In situ* hybridization of corticotropin-releasing factor-encoding messenger RNA in the hypothalamus of the white sucker, *Catostomus commersoni*. *Cell Tiss. Res.*, **267:** 545-549.

Olivereau, M., Moons, L., Olivereau, J., and Vandersande, F. (1988). Coexistence of corticotropin-releasing factor-like immunoreactivity and vasotocin in perikarya of the preoptic nucleus in the eel. *Gen. Comp. Endocrinol.*, **70:** 41-48.

Olivereau, M., and Olivereau, J. M. (1988*a*). Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. *Peptides,* **9:** 13-21.

Olivereau, M., and Olivereau, J. M. (1988*b*). Effect of hypophysectomy on the hypothalamic corticotropin-releasing factor (CRF) neuronal system in the eel: immunocytochemical study. *Neurosci. Let.,* **91:** 271-275.

Olivereau, M., and Olivereau, J. M. (1990*a*). Corticotropin-like immunoreactivity in the brain and pituitary of three teleost species (goldfish, trout and eel). *Cell Tiss. Res.,* **262:** 115-123.

Olivereau, M., and Olivereau, J. M. (1990*b*). Effect of pharmacological adrenalectomy on corticotropin-releasing factor-like and arginine vasotocin immunoreactivities in the brain and pituitary of the eel: immunocytochemical study. *Gen. Comp. Endocrinol.,* **80:** 199-215.

Olivereau, M., and Olivereau, J. M. (1991*a*). Corticotropin-like immunoreactivity in the brain of intact, hypophysectomised, cortisol- and metopirone-treated eels. *Cell Tissue Res.,* **265:** 485-492.

Olivereau, M., and Olivereau, J. M. (1991*b*). Responses of brain and pituitary immunoreactive corticotrophin-releasing factor in surgically interrenalectomized eels: immunocytochemical study. *Gen.Comp.Endocrinol.,* **81:** 295-303.

Ono, M., Wada, C., Oikawa, I., Kawazoe, I., and Kawauchi, H. (1988). Structures of two kinds of mRNA encoding the chum salmon melanin-concentrating hormone. *Gene,* **71:** 433-438.

Parkes, D. G., and Vale, W. (1993). Contrasting actions of melanin-concentrating hormone and neuropeptide-E-I on posterior pituitary function. *Proc. Natl. Acad. Sci. USA,* **680:** 588-590.

Perrot, M., Carrick, S., and Balment, R. (1991). Pituitary and plasma arginine vasotocin levels in teleost fish. *Gen. Comp. Endocrinol.,* **83:** 68-74.

Peter, R. E., and Gill, V. (1975). A stereotaxic atlas and technique for forebrain nuclei of the goldfish, *Carassius auratus*. *J. Comp. Neurol.*, **159:** 69-102.

Peter, R. E., and Fryer, J. N. (1983). 'Endocrine functions of the hypothalamus of actinopterygians.' In: *Fish Neurobiology*, pp. 165-201. Ed(s): R. E. Davis & R. G. Northcutt. Michigan: University of Michigan Press.

Peters, G. (1982). The effects of stress on the stomach of the European eel, *Anguilla anguilla* L. *J. Fish Biol.*, **21:** 497-512.

Peters, G., and Schwarzer, R. (1985). Changes in hemopoietic tissue of rainbow trout under influence of stress. *Dis. Aquat. Organisms,* **1:** 1-10.

Pickering, A. D. (1981). 'Introduction; the concept of biological stress.' In: *Stress and Fish*, pp. 1-9. Ed(s): A. D. Pickering. London: Academic Press.

Pickering, A. D. (1984). Cortisol-induced lymphocytopenia in brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.*, **53:** 252-259.

Pickering, A. D., and Duston, J. (1982). Administration of cortisol to brown trout, *Salmo trutta* L., and its effects on the susceptibility to *Saprolegnia* infection and furunculosis. *J. Fish Biol.*, **23:** 163-175.

Pickering, A. D., and Pottinger, T. G. (1983). Seasonal and diel changes in plasma cortisol levels of the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.*, **49:** 232-239.

Pickering, A. D., and Pottinger, T. G. (1987*a*). Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. *J.Fish Biol.*, 363-374.

Pickering, A. D., and Pottinger, T. G. (1987*b*). Crowding causes prolonged leucopenia in salmonid fish, despite interrenal acclimation. *J.Fish Biol.*, **30:** 701-712.

Pickering, A. D., Pottinger, T. G., and Christie, P. (1982). Recovery of brown trout, *Salmo trutta* L., from acute handling stress: a time-course study. *J. Fish Biol.*, **20:** 229-244.

Pickering, A. D., Pottinger, T. G., and Sumpter, J. P. (1986). Independence of the pituitary-interrenal axis and melanotroph activity in the brown trout, *Salmo trutta* L., under conditions of environmental stress. *Gen. Comp. Endocrinol.*, **64:** 206-211.

Pickering, A. G., Pottinger, T. G., and Sumpter, J. P. (1987). On the use of dexamethasone to block the pituitary-interrenal axis in the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.*, **65:** 346-353.

Pierson, P. M., Guibollini, M. E., and Lahlou, B. (1996). A V-1 type receptor for mediating the neurohypophysial hormone-induced ACTH release in trout pituitary. *J. Endocrinol.*, **149:** 109-115.

Plotsky, P. M. (1991). Pathways to the secretion of adrenocorticotropin: a view from the portal. *J. Neuroendocrinol.*, **3:** 1-9.

Plotsky, P. M., and Sawchenko, P. E. (1987). Hypophysial-portal plasma levels, median eminence content, and immunohistochemical staining of corticotropin-releasing factor, arginine vasopressin, and oxytocin after pharmacological adrenalectomy. *Endocrinol.*, **120:** 1361-1369.

Pottinger, T. G. (1991). The effect of stress and exogenous cortisol on receptor-like binding of cortisol in the liver of rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.*, **78:** 194-201.

Pottinger, T. G., and Brierley, I. (1997). A putative cortisol receptor in the rainbow trout erythrocyte: Stress prevents starvation-induced increases in specific binding of cortisol. *J. Exp. Biol.*, **200:** 2035-2043.

Pottinger, T. G., and Carrick, T. R. (1999). Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. *Gen. Comp. Endocrinol.*, **116:** 122-132.

Pottinger, T. G., and Moran, T. A. (1993). Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout (*Oncorhynchus mykiss*). *J. Fish Biol.*, **43:** 121-130.

Pottinger, T. G., and Pickering, A. D. (1992). The influence of social interaction on the acclimation of rainbow trout, *Onchorhnychus myklss* (Walbaum) to chronic stress. *J. Fish Biol.*, **41:** 435-447.

Pottinger, T. G., Prunet, P., and Pickering, A. D. (1992). The effects of confinement stress on circulating prolactin levels in rainbow trout (*Oncorhynchus mykiss*) in fresh water. *Gen.Comp.Endocrinol.*, **88:** 454-460.

Pottinger, T. G., Balm, P. H. M., and Pickering, A. D. (1995). Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. *Gen. Comp. Endocrinol.*, **98:** 311-320.

Powell, K. A., and Baker, B. I. (1987). Ultrastructural demonstration that melanin-concentrating hormone-like and alpha-melanocyte stimulating hormone-like immunoreactive molecules co-exist in the same neurosecretory granules. *Neurosci. Letters,* **80:** 268-274.

Presse, F., Hervieu, G., Imaki, T., Sawchenko, P. E., Vale, W., and Nahon, J.-L. (1992). Rat melanin-concentrating hormone messenger ribonucleic acid expression: marked changes during development and after stress and glucocorticoid stimuli. *Endocrinol.*, **131:** 1241-1250.

Quabius, E. S., Balm, P. H. M., and Wendelaar Bonga, S. E. (1997). Interrenal stress responsiveness of Tilapia (*Oreochromis mossambicus*) is impaired by dietary exposure to PCB 126. *Gen. Comp. Endocrinol.*, **108:** 472-482.

Rance, T. A., and Baker, B. I. (1979). The teleost melanin-concentrating hormone - a pituitary hormone of hypothalamic origin. *Gen. Comp. Endocrinol.*, **37:** 64-73.

Rance, T. A., and Baker, B. I. (1981). The *in vitro* response of the trout interrenal to various fragments of ACTH. *Gen.Comp.Endocrinol.*, **45:** 497-503.

Rance, T. A., Baker, B. I., and Webley, G. (1982). Variations in plasma cortisol concentrations over a 24-hour period in the rainbow trout *Salmo gairdneri*. *Gen. Comp. Endocrinol.*, **48:** 269-274.

Rand-Weaver, M., Pottinger, T. G., and Sumpter, J. P. (1993). Plasma somatolactin concentrations are elevated by stress. *Endocrinol.*, **138:** 509-515.

Rietveld, W. J. (1985). 'Functional significance of the suprachiasmatic nucleus.' In: *Circadian rhythms in the central nervous system*, pp. 45-55. Ed(s): P. H. Redfern, I. C. Cambell, J. A. Davies & K. F. Martin. London: Macmillian.

Rivier, C., and Vale, W. (1983). Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion *in vivo*. *Endocrinol.*, **113:** 939-942.

Rivier, C. L., and Plotsky, P. M. (1986). Mediation by corticotropin releasing-factor (CRF) of adenohyophyseal hormone secretion. *Ann. Rev.Physiol.*, **48:** 475-494.

Robertson, O. H., Hane, S., Wexler, B., and Rintret, A. P. (1963). The effect of hydrocortisone on immature rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.*, **3:** 422-436.

Roth, R. R. (1972). Some factors contributing to the development of fungus infection in freshwater fish. *J. Wildl. Dis.*, **8:** 24-28.

Rousseau, K., Le Belle, N., Marchelidon, J., and Dufour, S. (1999). Evidence that corticotropin-releasing hormone acts as a growth hormone-releasing factor in a primitive teleost, the European eel (*Anguilla anguilla*). *J. Neuroendocrinol.*, **11:** 385-392.

Ruane, N. M., Wendelaar-Bonga, S. E., and Balm, P. H. M. (1999). Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. *Gen. Comp. Endocrinol.*, **115:** 210-219.

Salbert, G., Chauveau, I., Bonnec, G., Valotaire, Y., and Jego, P. (1992). One of the two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol. Endocrinol.*, **6:** 1605-1613.

Sawchenko, P. E., Swanson, L. W., and Vale, W. W. (1984). Corticotropin-releasing factor: co-expression within distinct subsets of oxytocin-, vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat. *J. Neurosci.*, **4:** 1118-1129.

Sawchenko, P., Imaki, T., Potter, E., Kovacs, K., Imaki, J., and Vale, W. (1993). The functional neuroanatomy of corticotropin-releasing factor. *Ciba Foundation Symp.*, **172:** 5-29.

Sawyer, T. K., Hurby, V. J., Wilkes, B. C., Draelos, M. T., Hadley, M. E., and Bergsneider, M. (1982). Design and comparative biological activities of highly potent analogues of α-melanotropin$_{4-10}$. *J. Med. Chem.*, **25:** 1022-1027.

Sayers, G., and Portanova, R. (1974). Secretion of ACTH by isolated anterior pituitary cells: kinetics of stimulation by corticotropin-releasing factor and of inhibition by corticosterone. *Endocrinol.*, **94:** 1723.

Scaccianoce, S., Muscolo, L. A. A., Cigliana, G., Navarra, D., Nicolai, R., and Angelucci, L. (1991). Evidence for a specific role of vasopressin in sustaining pituitary-adrenocortical stress response in the rat. *Endocrinol.*, **128:** 3138-3143.

Schreck, C. B., Johnson, S. L., and Nickelson, T. E. (1989). Transportation stress affects performance of coho salmon, *Oncorhynchus kisutch*. *Aquacult.*, **82:** 15-20.

Seidah, N. G., Day, R., Marcinkiewicz, M., and Chretien, M. (1998). Precursor converatses; an evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. *Ann. N. Y. Acad. Sci.*, **839:** 9-24.

Shrimpton, J. M., and Randall, D. J. (1994). Downregulation of corticosteroid receptors in gills of coho salmon due to stress and cortisol treatment. *Am. J. Physiol.*, **36:** 432-438.

Sirinathsinghji, D. J. (1987). Inhibitory influence of corticotropin-releasing factor on components of sexual behaviour in the male rat. *Brain Res.*, **407:** 185-190.

Spencer, R. L., Kim, P. J., Kalman, B. A., and Cole, M. A. (1998). Evidence for mineralocorticoid receptor facilitation of glucocorticoid receptor-dependant regulation of hypothalamic-pituitary-adrenal axis. *Endocrinol.*, **139:** 2718-2726.

Spencer, R. L., and McEwan, B. S. (1990). Adaptation of the hypothalamo-pituitary-adrenal axis to chronic ethanol stress. *Neuroendocrinol.*, **52:** 481-489.

Suda, T., Tozawa, F., Yamada, M., Ushiyama, T., Tomori, T., Sumitomo, T., Nakagami, Y., Demura, H., and Shizume, K. (1988). Insulin-induced hypoglycemia increases corticotropin-releasing factor messenger ribonucleic acid levels in rat hypothalamus. *Endocrinol.*, **123:** 1371-1375.

Sumpter, J. P., Pickering, A. D., and Pottinger, T. G. (1985). Stress-induced elevation of plasma α-MSH and endorphin in brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.*, **59:** 257-265.

Sumpter, J. P., Dye, H. M., and Benfey, T. J. (1986). The effects of stress on plasma ACTH, α-MSH, and cortisol levels in salmonid fishes. *Gen. Comp. Endocrinol.*, **62:** 377-385.

Sutton, R. E., Koob, G. F., LeMoal, M., Rivier, J., and Vale, W. W. (1982). Corticotropin-releasing factor produces behavioural activation in rats. *Nature,* **297:** 331-333.

Suzuki, M., Baker, B. I., and Levy, A. (1996). 'Effects of tank colour and stress on melanin-concentrating hormone gene expression in the rainbow trout.' In: *The peptidergic neuron.*, pp. 279-284. Ed(s): B. Krisch & R. Mentlein. Berlin: Birkhausen Verlag.

Suzuki, M., Hyodo, S., and Urano, A. (1992). Cloning and sequence analysis of vasotocin and isotocin precursor cDNAs in the masu salmon, *Oncorhynchus masou*: Evolution of neurohypophysial hormone precursors. *Zool. Sci.*, **9:** 157-167.

Suzuki, M., Marnaware, Y. K., Baker, B. I., and Levy, A. (1995). Influence of environmental colour and diurnal phase on MCH gene expression in the trout. *J. Neuroendocrinol.*, **7:** 319-328.

Suzuki, M., Bennett, P., Levy, A., and Baker, B. I. (1997). Expression of MCH and POMC genes in rainbow trout (*Onchorhnychus myklss*) during ontogeny and in response to early physiological challenges. *Gen.Comp.Endocrinol.*, **107:** 341-350.

Swanson, L. W., Sawchenko, P. E., Rivier, J., and Vale, W. W. (1983). Organisation of ovine corticotropin-releasing factor immunoreactive cells and fibres in the rat brain: an immunohistochemical study. *Neuroendocrinol.*, **36:** 165-186.

Swanson, L. W., and Simmons, D. M. (1989). Differential steroid hormone and neural influences on peptide mRNA levels of CRH cells of the paraventricular nucleus: a hybridisation histo-chemical study in the rat. *J. Comp. Neurol.*, **285:** 413-435.

Swift, D. J. (1982). Changes in selected blood component values of rainbow trout, *Salmo gairdneri* Richardson, following the blocking of the cortisol stress response with betamethasome and subsequent exposure to phenol or hypoxia. *J. Fish Biol.*, **21:** 269-277.

Takebe, K., Sakakura, M., and Mushimo, K. (1972). Continuance of diurnal rhythmicity of CRF activity in hypophysectomised rats. *Endocrinol.*, **126:** 1904-1913.

Tannahill, L. A. (1987). 'Neurohormonal control of adrenocorticotrophin synthesis and release.' University of Edinburgh, Edinburgh.

Taylor, A. D., Christian, H. C., Morris, J. F., Flower, R. J., and Buckingham, J. C. (1997). An antisense oligodeoxynucleotide to lipocortin 1 reverses the inhibitory actions of

dexamethasone on the release of adrenocorticotropin from rat pituitary tissue *in vitro*. *Endocrinol.*, **138:** 2909-2918.

Teitsma, C. A., Bailhache, T., Tujague, M., Balment, R. J., Ducouret, B., and Kah, O. (1997). Distribution and expression of glucocorticoid receptor mRNA in the forebrain of rainbow trout. *Neuroendocrinol.*, **66:** 294-304.

Teitsma, C. A., Anglade, I., Toutirais, G., Munzo-Cueto, J.-A., Saligaut, D., Ducouret, B., and Kah, O. (1998). Immunohistochemical localisation of glucocorticoid receptors in the forebrain of rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Neurol.*, **401:** 395-410.

Urano, A., Hyodo, S., and Suzuki, M. (1992). Molecular evolution of neurohypophysial hormone precursors. *Prog.Brain Res.*, **92:** 39-46.

Vale, W., Vaughan, J., Smith, M., Yamamoto, G., Rivier, J., and Rivier, C. (1983). Effects of synthetic ovine corticotropin-releasing factor, glucorticoids, catecholamines, neurohypophyseal peptides, and other substances on cultured corticotropic cells. *Endocrinol.*, **113:** 1121-1131.

Van de Pavert, S. A., Clarke, I. J., Rao, A., Vrana, K. E., and Schwartz, J. (1997). Effects of vasopressin and elimination of corticotropin-releasing hormone-target cells on pro-opiomelanocortin mRNA levels and adrenocorticotropin secretion in ovine anterior pituitary cells. *J. Endocrinol.*, **154:** 139-147.

Van den Dungen, H., Buijs, R., Pool, C., and Terlou, M. (1982). The distribution of vasotocin and isotocin in the brain of rainbow trout. *J.Comp.Neurol.*, **212:** 146-157.

Van Enckevort, F. H. J., Pepels, P. P. L. M., Leunissen, J. A. M., Martens, G. J. M., Wendelaar-Bonga, S. E., and Balm, P. H. M. (2000). *Oreochromis mossambicus* (tilapia) corticotropin-releasing hormone: cDNA sequence and bioactivity. *J. Neuroendocrinol.*, **12:** 177-186.

Vijayan, M. M., Pereira, C., Grau, E. G., and Iwama, G. K. (1997). Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comp. Biochem. Physiol. C,* **116:** 89-95.

Wand, G. S., and Eipper, B. A. (1987). Effect of chronic secretagogue exposure on pro-adrenocorticotropin/endorphin production and secretion in primary cultures of rat anterior pituitary. *Endocrinol.,* **120:** 953-961.'

Warne, J. M., and Balment, R. J. (1997). Vascular actions of neurohypophysial peptides in the flounder. *Fish Physiol. Biochem.,* **17:** 313-318.

Watabe, T., Tanaka, K., Kumagae, M., Itoh, S., Hasegawa, M., Horiuchi, T., Miyabe, S., Ohno, H., and Shimizu, N. (1987). Diurnal rhythm of plasma immunoreactive corticotrophin-releasing factor in normal subjects. *Life Sci.,* **40:** 1651-1655.

Watts, A., and Swanson, L. (1989). Diurnal variation in the content of preprocorticotrophin-releasing hormone messenger ribonucleic acids in the hypothalamic paraventricular nucleus of rats of both sexes as measured by *in situ* hybridisation. *Endocrinol.,* **125:** 1734-1738.

Wedemeyer, G. A., Gould, R. W., and Yasutake, W. T. (1983). Some potentials and limits of the leucocrit test as a fish health assessment method. *J. Fish Biol.,* **23:** 711-716.

Wendelaar-Bonga, S. E. (1993). 'Endocrinology.' In: *The Physiology of Fishes*, pp. 469-490. Ed(s): D. H. Evans. Boca Raton, Florida: CRC Press.

Wendelaar-Bonga, S. E. (1997). The stress response in fish. *Physiol.Rev.,* **77:** 591-625.

Wendelaar-Bonga, S. E., Balm, P. H. M., and Lamers, A. E. (1995). The involvement of ACTH and MSH in the stress response in teleost fish. *Netherlands J. Zool.,* **45:** 103-106.

Wendelaar-Bonga, S. E., and Lock, R. A. C. (1992). Toxicants and osmoregulation in fish. *Netherlands J. Zool.,* **42:** 478-493.

Whitnall, M. H. (1988). Distribution of pro-vasopressin expressing and pro-vasopressin deficient CRH neurons in the paraventricular hypothalamic nucleus of colchicine-treated animals. *J.Comp.Neurol.*, **275:** 13-28.

Whitnall, M. H. (1989). Stress selectively activates vasopressin containing subset of corticotropin-releasing hormone neurosecretory cells. *Neuroendocrinol.*, **50:** 702-707.

Whitnall, M. H. (1990). Subpopulation of corticotropin-releasing hormone neurosecretory cells distinguished by presence or absence of vasopressin: confirmation with multiple corticotropin-releasing hormone antisera. *Neuroscience,* **36:** 201-205.

Whitnall, M. H., Smith, D., and Gainer, H. (1987). Vasopressin coexists in half of the corticotropin-releasing factor axons present in the external zone of the median eminence in normal rats. *Neuroendocrinol.*, **45:** 420-424.

Widmaier, E. P., and Dallman, M. F. (1984). The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perifused pituitaries *in vitro*: rapid inhibition by glucocorticoids. *Endocrinol.*, **115:** 2368-2374.

Wilkinson, C. W., Shinsako, J., and Dallman, M. F. (1979). Daily rhythms in adrenal responsiveness to adrenocorticotrophin are determined primarily by the time of feeding in the rat. *Endocrinol.*, **104:** 350-359.

Willemse, J. J., Markus-Silvis, L., and Ketting, G. H. (1984). Morphological effects of stress in cultured elvers, *Anguilla anguilla* (L.). *Aquaculture,* **36:** 193-201.

Winberg, S., Carter, C. G., McCarthy, I. D., He, Z.-Y., Nilsson, G. E., and Houlihan, D. F. (1993). Feeding rank and brain serotonergic activity in rainbow trout *Oncorhynchus mykiss. J. Exp. Biol.*, **179:** 197-211.

Wolfson, B., Manning, R. W., Davis, L. G., Arentzen, R., and Baldrino Jr., F. (1985). Co-localisation of corticotropin-releasing factor and vasopressin mRNA in neurones after adrenalectomy. *Nature,* **315:** 59-61.

Young, E. A., Kwak, S. P., and Kottak, J. (1995). Negative feedback regulation following administration of chronic exogenous corticosterone. *J. Neuroendocrinol.*, **7:** 37-45.

Yulis, C. R., and Lederis, K. (1987). Co-localization of the immunoreactivities of corticotropin-releasing factor and arginine vasotocin in the brain and pituitary system of the teleost *Catostomus commersoni*. *Cell Tiss. Res.*, **247:** 267-273.

Zupanc, G. K. H., Horschke, I., and Lovejoy, D. A. (1999). Corticotrophin releasing factor in the brain of the gymnotiform fish, *Apteronotus leptorhynchus* : immunohistochemical studies combined with neuronal tract tracing. *Gen. Comp. Endocrinol.*, **114:** 349-364.